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THE UNIVERSITY OF ALBERTA

Behavioral, Pharmacologic and Biochemical Studies of a Fluorinated Analogue of Amphetamine in Mice and Rats

by

Rae Keashly

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Master of Science

IN

Pharmaceutical Sciences (Toxicology)

Faculty of Pharmacy and Pharmaceutical Sciences

EDMONTON, ALBERTA

FALL 1987

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Behavioral, Pharmacologic and Biochemical Studies of a Fluorinated Analogue of Amphetamine in Mice and Rats submitted by Rae Keashly in partial fulfilment of the requirements for the degree of Master of Science in Pharmaceutical Sciences (Toxicology).

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Supervisor

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Date... Sept. 28, 1987.....

ABSTRACT

The compounds studied were the enantiomers of α-fluoromethyl-β-phenethylamine (FAM), a fluorinated analogue of amphetamine (AM).

Mice treated with R-(+)- or S-(-)-FAM exhibited prolonged, dose-dependent decreases in both locomotor activity and rectal temperatures. Only mice treated with a high dose of R-(+)-FAM showed a biphasic locomotor response consisting of a transient increase in locomotor activity 30 min after treatment followed by a prolonged period of decreased locomotor activity. R-(-)-AM had little effect on murine rectal temperature but induced a transient decrease in locomotor activity. S-(+)-AM induced increased rectal temperatures in mice followed by a slight, transient decrease. S-(+)-AM also induced increased locomotor activity followed by decreased locomotor activity. Neither the temperature nor the locomotor decreases induced by AM was dose-dependent. The periods of decreased locomotor activity induced by FAM were accompanied by light sedation. This was in contrast to AM, with which decreased locomotor activity was accompanied by stereotypic behaviors rather than light sedation.

In rats, both R-(+)-FAM and S-(+)-AM increased rectal temperatures. However, the temperature effect of R-(+)-FAM could be prevented by pretreatment with phenobarbital while that of S-(+)-AM was unaffected by pretreatment.

In rats, brain levels of R-(+)-FAM and S-(+)-AM were similar after equimolar doses and declined in a log-linear manner. In mice, S-(+)- or R-(-)-AM levels were higher than those of R-(+)- or S-(-)-FAM. In addition, AM levels exhibited a log-linear decline while FAM levels exhibited a more rapid, biphasic decline. Further, FAM levels were dependent upon the enantiomer and the sex and strain of the mice.

Urinary excretion of unchanged R-(+)- or S-(-)-FAM (24 h) in mice was lower than that of S-(+)- or R-(-)-AM. This was also true in rats, although rats excreted more unchanged FAM than did mice. When rats were pretreated with phenobarbital the urinary excretion of unchanged R-(+)- and S-(-)-FAM and R-(-)-AM decreased while the excretion of S-(+)-AM was unaffected.

It appears that the enantiomers of FAM are more rapidly metabolized by mice than those of AM and that rats metabolize R-(+)-FAM by a route different from that of S-(+)-AM.

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LIST OF ABBREVIATIONS

X°	— X degrees celsius
β-FAM	— β-fluoroamphetamine
β,β-DFAM	— β,β-difluoroamphetamine
β,β-DFpCA	— β,β-difluoro-*p*-chloroamphetamine
β,β-DFPEA	— β,β-difluoro-β-phenylethylamine
μL	— microlitre(s)
μm	— micrometre(s)
μmol	— micromole(s)
AM	— amphetamine
APC	— apparent partition coefficient
CI	— chemical ionization
CNS	— central nervous system
COMT	— catechol-*O*-methyltransferase
CRC	— Chemical Rubber Company
DA	— dopamine
DPEA	— 2,4-dichloro-6-phenylphenoxyethylamine
ECD	— electron capture detector
FAM	— α-fluoromethyl-β-phenylethylamine
FdUMP	— 5-fluoro-2'-deoxyuridine-5'-phosphate
FIP	— fluorinated isoproterenol
FNA	— fluorinated adrenaline
FpCA	— α-fluoromethyl-β-p-chlorophenylethylamine
FPE	— fluorinated phenylephrine
g	— gram(s)
X g	— times gravity
GABA	— γ-aminobutyric acid

GABA-T	— γ-aminobutyric acid transaminase
GC	— gas chromatograph(y,ic)
h	— hour(s)
5-HIAA	— 5-hydroxyindole acetic acid
5-HT	— 5-hydroxytryptamine (serotonin)
ID	— internal diameter
i.p.	— intraperitoneal
IP	— isoproterenol
kg	— kilogram(s)
L	— litre(s)
L-DOPA	— L-3,4-dihydroxyphenylalanine
M	— molar
MAO	— monoamine oxidase
MAO-A	— monoamine oxidase, type A
MAO-B	— monoamine oxidase, type B
min	— minute(s)
mg	— milligram(s)
mL	— millilitre(s)
mm	— millimeter(s)
mol	— mole(s)
MS	— mass spectrometry
N	— normal
NA	— noradrenaline
ng	— nanogram(s)
p	— probability
pCA	— p-chloroamphetamine
PE	— phenylephrine
PEA	— phenylethylamine

PFBC	— pentafluorobenzoyl chloride
S.E.M.	— standard error of the mean
SKF-525A	— β-diethylaminoethyl diphenylpropyl acetate
TFAM	— α-trifluoromethyl-β-phenylethylamine
TPC	— total partition coefficient
TYR	— tyramine

I. Introduction

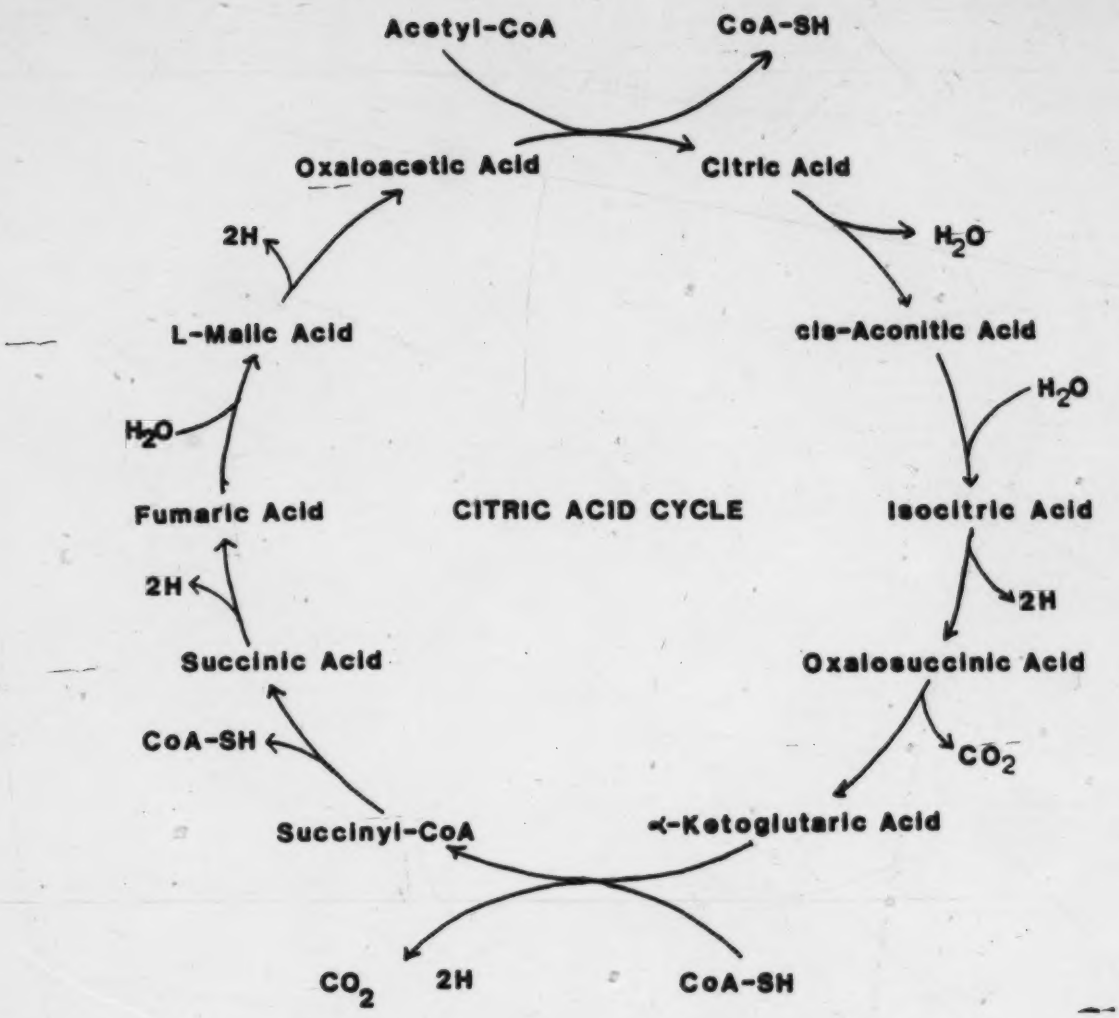
The first researcher to synthesize an organo-fluorine compound was Swarts who in 1896 synthesized fluoroacetic acid.[78] Interest in organic fluorine chemistry, and fluoroacetic acid in particular, increased during World War II when fluoroacetic acid was identified as the toxic principle of a South African plant involved in cattle deaths. This compound served as a prototype for development of a series of organo-fluorine pesticides and war gases.[64,68] It has since been demonstrated that fluoroacetic acid is the active toxic principle of at least 36 plants[64].

Another step forward in the understanding of organo-fluorine compounds was the discovery by Peters and Martius[64] that the toxicity of fluoroacetic acid was due to inhibition of the citric acid cycle. They observed that fluoroacetic acid was converted *in vivo* to fluorocitrate which irreversibly blocked the enzyme aconitase, thus preventing the conversion of citrate to aconitate and locking the citric acid cycle at that step (Figure I-1). This *in vivo* production of a toxic metabolite was termed "lethal synthesis" by Peters[64].

Other organo-fluorine compounds have since been synthesized and interest in this field has been sufficient to result in several symposia dedicated to the discussion of their unusual properties. Collections of documents arising from three of these symposia were published in 1971[20], 1976[21] and 1982[23]. It is interesting to note the change in emphasis from the toxicity of organo-fluorine compounds in 1971 to their biochemistry in 1976 and their biomedical applications in 1982. These compounds are now widely used in medicine (Table I-1) as CNS agents, general anaesthetic agents, antiarthritics, antitumour and antiviral agents. Of major current interest is the high solubility of oxygen in perfluorochemical emulsions and their application as artificial blood.[67,81]

A. Physico-chemical Aspects of the Carbon-Fluorine Bond

The diversity of biomedical applications of organo-fluorine compounds leads one to wonder what characteristics of fluorine and the carbon-fluorine bond have resulted in the widespread use of fluorination as a means of altering biological properties. Schlosser[69] has



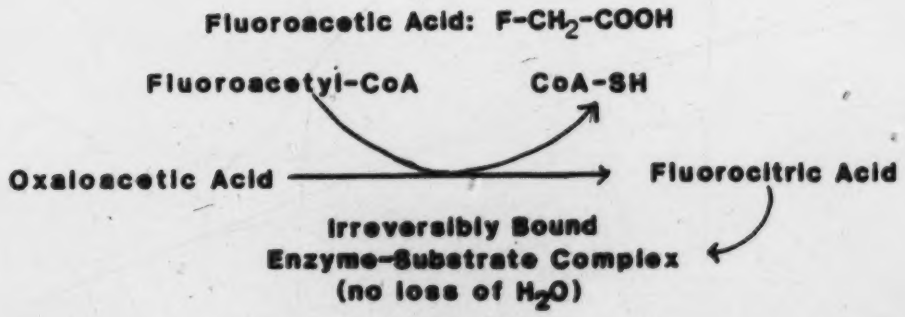


Figure I-1: Citric acid cycle blockade by fluoroacetic acid (after Schlosser[69]).

Table I-1: Current medical applications of fluorinated organic compounds.[22,23]

Application	Examples
CNS-active agents	Haloperidol
	Flufenazine
	Fenfluramine
Anti-tumour and anti-viral agents	5-Fluorouracil
	Ftorafur
	2'-Fluoro-2'-deoxy-arabinosyl—5-methyl-uracil
	Trifluridine
Steroids	9α-Fluorocortisol
	Dexamethasone
Non-Steroidal Antiinflammatory Drugs	Flufenisal
	Diflufenisal
	Sulindac
	Flurbiprofen
Non-acidic NSAIDs	Flazalone
	Floctafenine
Anaesthetics	Halothane
	Methoxyflurane
	Enflurane
	Isoflurane
Antibiotic and Antifungal Agents	Floxacillin
	5-Fluorocytosine
	3-Fluoro-D-alanine
Diuretics and Antihypertensive Agents	Bendroflumethiazide
	Polythiazide
	Triflocin

remarked "what is special about fluorine [is] nothing else than its position in the periodic table" suggesting that the small atomic radius of fluorine along with its strong electron-withdrawing property play important roles in its effect upon biological properties.

Fluorine is the most electronegative (electron-attracting) element in the periodic table.[10] This property causes the the electrons to be held close to the fluorine nucleus, resulting in an atom not much larger than hydrogen (the smallest atom in the periodic table)(Table I-2). This electron-withdrawing property also affects bonding electrons, pulling them toward the fluorine and resulting in C-F bonds which are similar to C-H bonds in strength. The aliphatic C-F bond tends to be resistant to most displacement reactions.[62,70] In an SN1 reaction the rate limiting step would be the cleavage of the C-F bond to form a cation which would then be attacked by a nucleophile.[62] However, the high energy of the C-F bond makes this cleavage unlikely. It would seem reasonable to suppose that a carbon attached to fluorine would be particularly susceptible to an SN2 attack as the carbon would have a slightly positive charge and therefore be open to nucleophilic attack.[70] However, the transition state of an SN2 reaction would require the C-F bond to "stretch" as the nucleophile approached the carbon. As fluorine is not easily polarizable, this "stretching" would not occur and the fluorine would remain relatively close to the carbon. This short C-F bond would permit the slightly negative fluorine to repel the approaching nucleophile, thus preventing it from coming close enough to the carbon to complete the transition arrangement.[62,70] However, fluorine displacement by an SN2 mechanism can be facilitated in an aqueous system by the presence of acid. An example is the hydrolysis of benzyl fluoride which is catalyzed by acid. This appears to be due to the formation of a hydrogen bond between the fluorine and the hydronium ion during the transition state.[70] The strength of the hydrogen bond with the fluorine permits the C-F bond to "stretch" and allows the nucleophile (in this case water) to approach the carbon without being repelled by the fluorine.[62]

Usually a single fluorine in an aromatic ring is also resistant to displacement, but electron-withdrawing groups (*e.g.* NO_2) *ortho* or *para* to the fluorine will activate the fluorine, permitting nucleophilic displacement.[62,63]

Table I-2: Physico-chemical properties of fluorine and the carbon-fluorine bond (adapted from Hugheey[45])

X	van der Waal Radius (nm)	C-X Bond Length (nm)	C-X Bond Strength (kJ/mol)	Electronegativity
H	1.2-1.45	10.9	411	2.20
OH	1.5	14.3	358	3.5
F	1.5-1.6	13.5	485	4.1
Cl	1.7-1.9	17.7	327	2.83
Br	1.8-2.0	19.4	285	2.74
I	1.95-2.12	21.4	213	2.21

The small size of the fluorine atom allows it to substitute for hydrogen in a molecule with little or no strain or distortion. This makes it possible for a fluorinated compound to mimic its hydrogen analogue at even those receptor sites with rigid steric requirements. However, the electronegativity of fluorine may alter the electronic structure of a molecule to such an extent that the molecule may no longer be accepted or active at receptors with rigid electronic requirements. An electronic alteration observed when fluorine is substituted into acids or bases is a change in pKa (Table I-3). This alters the proportion of neutral and charged species present at physiological pH. Fluorine substitution also increases the likelihood of hydrogen bonding and dipole interactions[10,23,69,75].

A biological effect often attributed to fluorine substitution is increased lipophilicity[15,22,53]. The references generally quoted to support this claim [25,41] deal mostly with the effect of substitution of fluorine and the trifluoromethyl moiety ($-CF_3$) into aromatic rings. CF_3 does appear to enhance the lipophilicity of these molecules as do the few examples of simple fluorine substitution into aromatic rings. However, little work appears to have been done on the lipophilic effects of aliphatic fluorine substitution. It is probably more accurate to state that while fluorine substitution usually results in increased lipophilicity, it is not axiomatic[24]. For example, 1-fluoropentane is ten times less lipid-soluble than is pentane.[42] If fluorination does result in an analogue which is more lipid-soluble than the parent compound, this increase in lipophilicity would result in increased rates of absorption and diffusion through cellular membranes *in vivo*. An increased lipid solubility could also increase the volume of distribution of the fluoro analogue compared to the parent compound by enabling a larger proportion of the fluoro analogue to be absorbed into fatty tissues away from the systemic circulation.

It was originally thought that the high bond energy of the C-F bond would result in metabolic stability.[68,75] In partial refutation of this hypothesis it was discovered that fluoroacetate, methoxyflurane and 4-fluoroacetanilide could be defluorinated by enzymatic means both *in vivo* and *in vitro*.[75] In fact, when fluorine is attached to a metabolically active site it can be a good leaving group[57,69], as illustrated by the increased rate of dehalogenation

Table–I-3: Effect of fluorination on pKa of acids and bases.

Acid	pKa	Base	pKa
Acetic acid	4.7	Ethylamine	10.8†
Trifluoroacetic acid	0.23	$\beta\beta\beta$-Trifluoroethylamine	5.7†
Benzoic acid	4.2	Aniline	4.6
o-Fluorobenzoic acid	2.9	2-Fluoroaniline	3.2
		3-Fluoroaniline	3.5

Values from CRC Handbook[84] except † from Hugheey.[45]

of fluoroacetate as compared to chloroacetate by haloacetate halidohydrolase (obtained from a psuedomonad).[40] When in an area adjacent to an active site, the electron-withdrawing effect of fluorine can also alter metabolic reactions (see discussion of amphetamine *vs* β,β-difluoroamphetamine). Thus the location of the fluorine atom in the molecule of interest is also important in determining the differences in biology and biochemistry observed between a compound and its fluorinated analogue.

B. Adrenocorticoids

Depending upon the location of fluorine in a drug molecule, a fluorinated drug may have enhanced, attenuated or equivalent pharmacological properties compared to the hydrogen analogue. An early example of this manipulation of pharmacological properties is the fluorination of steroids. Proper positioning of the fluorine in a steroid can alter the balance between glucocorticoid, antiinflammatory and mineralocorticoid activities. That is, it becomes possible to separate the multiple pharmacological properties of steroids by selective fluorination. This is illustrated by a number of studies reviewed by Wettstein[85].

Pharmacological evaluations of cortisol analogues (Fig. I-2) have enabled researchers to establish some structure-activity relationships of fluorine substitution. The glycogenic, anti-inflammatory and sodium retention activities of cortisol are all increased by 9α-fluorination which increases the acidity of the 11β-hydroxyl group. In cortisol, the 11β-hydroxyl group is sterically hindered, but the increased acidity could increase the hydrogen bonding between the hydroxyl and a nucleophilic group in the receptor site, permitting a "firmer" bond between the cortisol analogue and the receptor.[85] In addition, it may be that fluorine itself forms hydrogen bonds with electrophilic groups (*e.g.* amino or hydroxyl groups) in the active site, and this would also increase the interaction between the receptor site and the ligand.[85] 12α-Fluorination would also be expected to increase the acidity of the 11β-hydroxyl group and thereby increase the biological activity of cortisol. This is not the case as the 12α-fluorine forms a hydrogen bond with the 17α-hydroxyl group, resulting in decreased glucocorticoid activity compared to the 9α-fluoro analogue while maintaining an equivalent

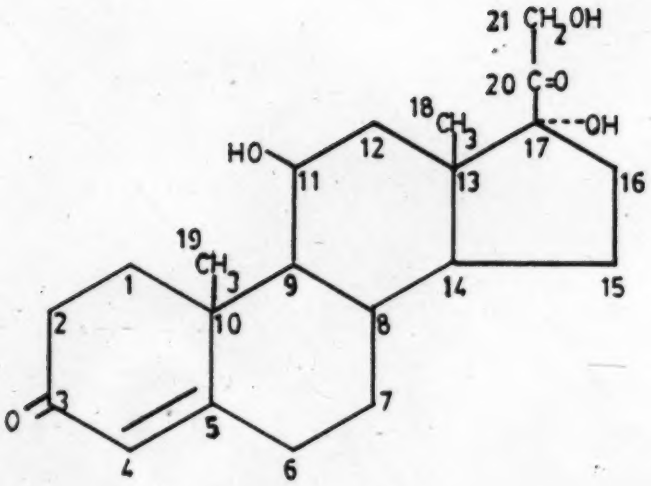


Figure I-2: Structure of cortisol.

sodium retention activity. If hydrogen bonding is prevented, then the 12α-fluoro analogue of cortisol has activity similar to that of the 9α-fluorinated analogue.[85]

6α-Fluorination increases the glycogenic and anti-inflammatory activities relative to cortisol but decreases the mineralocorticoid activity. This alteration in activity is not easily explained by the properties of the molecule. Fluorination at the 6 carbon in the cortisol structure destabilizes the 4-5 double bond, thereby increasing the susceptibility to electrophilic reactions.[85] This results in increased metabolism of the 6α- and 6β-fluorocortisols by liver enzymes. The increased metabolism may account for the low biological activity of the 6β-analogue but does not explain the increased activities observed with the 6α- analogue. In this case, it may not be the alteration of electronic properties of the cortisol molecule which is responsible for the activity of 6α-fluorocortisol. This possibility is supported by the observation that 6α-methylation results in biological activities similar to those of the 6α-fluoro analogue even though the methyl group is an electron-donating substituent and would not alter the electronic properties of cortisol in the same manner as would fluorine.[85]

Attempts to retain the high anti-inflammatory activity of the 9α-fluoro analogue of cortisol, while reducing the sodium retention side effect led to the use of multiple fluorinations.[85] 6α-Fluorination is not enough to overcome the sodium retention induced by 9α-fluorination, but the 6α,9α,16α-trifluorinated analogue of cortisol has decreased mineralocorticoid properties relative to cortisol, with anti-inflammatory activity higher than that observed after 9α-fluorination alone.[85]

C. Ring-fluorinated Biogenic Amines

Ring fluorination of biogenic amines (Fig. I-3) has been examined by Kirk, Cantacuzene *et al.*[8,50-52,61] in order to establish how changes in the pKa of the phenol substituents might alter the biochemical and pharmacological properties of these compounds. They observed that the introduction of fluorine into the aromatic ring decreases the pKa of noradrenaline (NA), dopamine (DA), tyramine (TYR) and serotonin (5-HT) (Table I-4). The authors attributed this decrease to an increase in the acidity of the phenol groups rather

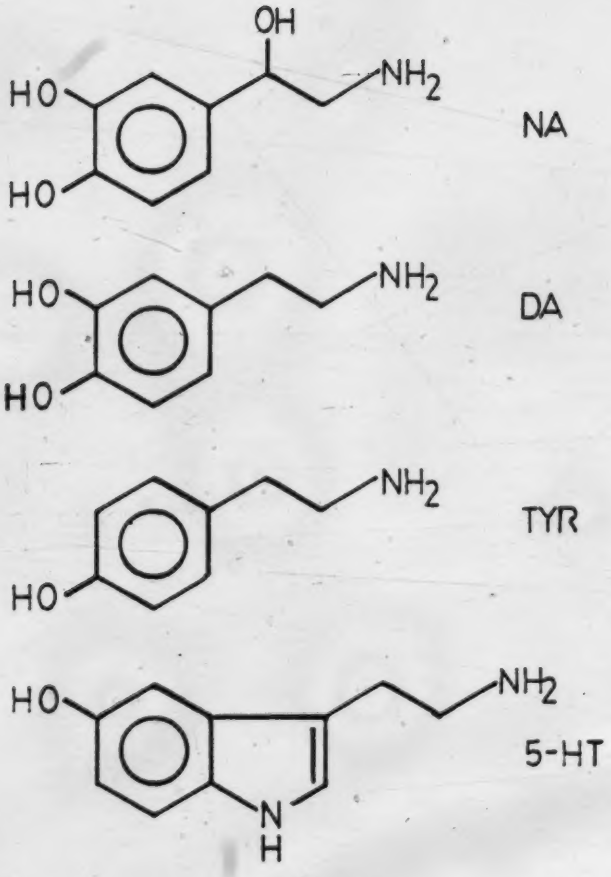


Figure I-3: Structures of biogenic amines.

Table I-4: Effect of ring-fluorination on the pKa of biogenic amines (after Kirk *et al.*[50]).

Amine	pKa	Amine	pKa
Serotonin	10.7	Noradrenaline	8.9
4,6-Difluoroserotonin	8.0	2-Fluoronoradrenaline	7.7
Tyramine	9.5	5-Fluoronoradrenaline	7.8
3-Fluorotyramine	8.4	6-Fluoronoradrenaline	8.5
3,5-Difluorotyramine	7.0	Dopamine	8.9
		2-Fluorodopamine	8.0
		5-Fluorodopamine	8.0
		6-Fluorodopamine	8.5

than a decrease in the basicity of the amine groups. In addition, they felt that ring-fluorination would increase the degree of phenol ionization at physiological pH. Biochemically, ring fluorination alters the metabolism of biogenic amines by MAO and COMT. So, whereas 5-HT is a relatively poor substrate for MAO-B (Km ≈ 1000 μM), 4,6-difluoro 5-HT is a very good substrate (Km = 50 μM). Similarly, ring fluorination of TYR also increases metabolism by MAO-B: TYR (Km ≈ 170 μM), 3-fluoro TYR (Km = 18 μM) and 3,5-difluoro TYR (Km = 25 μM)[50].

Studies with COMT showed that ring fluorination alters the ratio of *para- vs meta*-O-methylation of NA. While NA exhibits a 9:1 preference for 3-O methylation, fluorination at the 5 position in the aromatic ring increases 4-O methylation such that 5-fluoro NA does not exhibit any significant preference for 3-O methylation. Conversely, 2- and 6-fluoro NA exhibit increased preference for 3-O-methylation compared to NA. The investigators interpreted these observations to indicate that O-methylation of NA by COMT proceeds *via* the phenolate anion, the proportion of which is increased by fluorine substituents *ortho* or *para* to the hydroxyl group undergoing methylation.

Effects of ring-fluorination on receptor affinity of DA and NA have also been examined[8]. These studies demonstrate that 6-fluoronoradrenaline (6-FNA) retains the ability of NA to displace radiolabeled ligands from *alpha*-receptors but has a decreased ability to displace ligands from *beta*-receptors. This suggests that 6-fluorination alters the receptor affinity of NA to that of pure *alpha* specificity. 2-Fluorination has the opposite effect, *i.e.* *alpha* affinity is lost but *beta* affinity is retained. On the other hand, 5-fluoronoradrenaline (5-FNA) affinity is similar to that of NA. In contrast to these effects on NA, the receptor affinities of DA were not modified by ring fluorination. *In vivo* and *in vitro* studies of the agonist properties of the ring-fluorinated analogues of NA showed alterations similar to those observed in the receptor affinity studies. That is, 5-FNA is equipotent to NA as an α- and β-agonist, while 2-fluoronoradrenaline (2-FNA) is a pure β-agonist and 6-FNA is a pure α-agonist.[8]

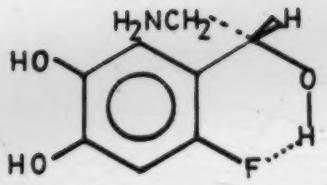
It is interesting to compare the agonist studies to the COMT studies. In the COMT studies 2-FNA and 6-FNA are both similar to NA, yet in the affinity and agonist studies these two analogues vary markedly from NA and each other. Cantacuzene *et al.*[8] felt that this indicated that alterations in the phenolic character of NA is not the major cause of the agonist and affinity alterations as the phenolic groups of both analogues would be perturbed to the same extent as supported by the COMT studies. The authors suggested that the agonist specificity could be due to configurational changes in the molecules brought about by hydrogen bonding between the fluorine and the hydrogen of the aliphatic hydroxyl group (Fig. I-4). Such an interaction could result in the preference of one rotamer over the other. Further, the preferred rotamer for 2-FNA (responsible for α-agonist activity) would be different from that preferred by 6-FNA (responsible for β-agonist activity)(Fig. I-4).

Studies with isoproterenol (IP) (a specific β-adrenergic agonist) and phenylephrine (PE) (a specific α-adrenergic agonist) support this hypothesis. The β-agonist activity of IP is maintained with 2-fluorination but lost with 6-fluorination; the reverse is true for PE where 2-fluorination abolished the α-agonist activity while 6-fluorination retained the α-agonist activity of the parent compound. In both cases the active fluoro analogue would have a preferred rotamer equivalent to the similarly active fluorinated NA (Fig. I-4). The effect of ring-fluorination is to maintain or lose the activity at one receptor rather than increase activity at the alternate receptor. This effect has been termed "the negative fluorine effect"[8].

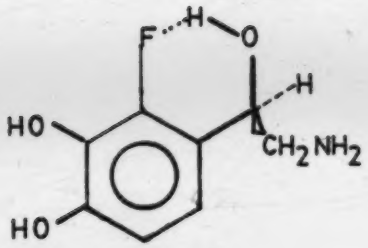
An interesting parallel therefore appears to exist between NA and steroids. In both cases fluorination can serve to separate multiple pharmacological or biochemical properties through effects upon the receptor-ligand interaction by altering either an electronic effect (steroids) or a conformational preference (NA).

D. "Suicide Substrates"

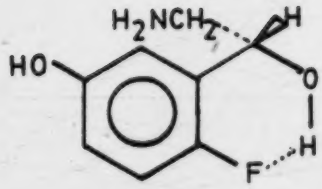
Certain fluorinated analogues of enzyme substrates such as fluorocitrate and FdUMP have been shown to be active antimetabolites, that is they interfere with the metabolism of naturally-occurring substrates[40,54]. These fluoro analogues were usually designed for use as



6-FNA

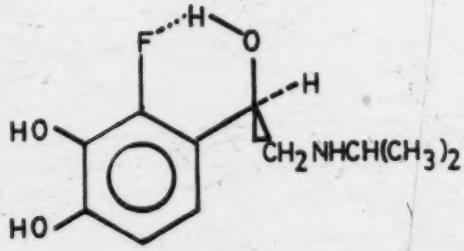


2-FNA



6-FPE

α-Agonist
Specific



6-FIP

β-Agonist
Specific

Figure I-4: Preferred rotamers of ring-fluorinated biogenic amines (after Kirk *et al.*[50]).

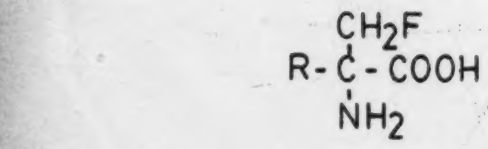
pharmacologic and metabolic probes rather than for use as medicinal agents, although some of these studies have produced drugs currently in use[40,54]. In 1970, Kollonitsch[54-57] used fluorination to "ration[ally] design" a wide spectrum antibiotic, 3-fluoro-D-alanine. This compound turned out to be an irreversible inhibitor of bacterial alanine racemase, a pyridoxal-dependent enzyme necessary for the production of bacterial cell walls. This work lead to the development of a general approach to the production of antimetabolites which is to maximally alter the chemical (*i.e.* electronic) character of the natural substrate or product while maintaining the geometry, a process termed "isogeometric modification"[54]. This would result in an antimetabolite that is "sterically acceptable" to the target enzyme but different enough chemically to interfere with the enzymic process[54]. This approach is almost tailor made for fluorine substitution and resulted in the synthesis of a number of fluorinated analogues of enzyme substrates (*e.g.* amino acids) and enzyme products (*e.g.* amines) which irreversibly inhibit specific pyridoxine-dependent enzymes (Table I-5).

The proposed method of enzyme inhibition[57] (Figure I-5) involves the formation of a Schiff base between the pyridoxal phosphate cofactor and the fluorinated analogue at the target enzyme's active site. This is followed by the loss of CO_2 (if the inhibitor is an amino acid) and the elimination of fluoride. The resulting imine is open to nucleophilic attack by an enzyme residue such as the terminal amino group of lysine, present in the active site of the enzyme. This irreversible binding of the substrate/inhibitor to the catalytic site of the enzyme results in irreversible deactivation.[54-57] Work by Silverman *et al.*[71-74] on S-4-amino-5-fluoro-pentanoic acid shows a change in absorption spectra during the inhibition process, indicating the cofactor is converted to the pyridoxamine phosphate form. Thus, inactivation only occurs when the enzyme actually acts upon the substrate, thereby destroying the substrate and inactivating itself. Compounds capable of inhibiting enzymes in this manner have been termed "suicide substrates"[1]. In the general structures shown in Table I-5, it is the R group which is responsible for the selectivity of the inhibitor[54]. For example, S-α-fluoromethyl-DOPA is a potent inhibitor of aromatic amino acid decarboxylase but has no effect on mammalian histidine decarboxylase while the reverse is true of S-α-fluoromethyl-histidine. In addition,

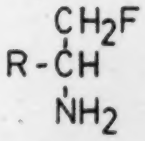
Table 1-5: Some examples of "suicide substrates" containing the α-fluoromethyl group (after Kollonitsch *et al.*[57]).

Compound	Inhibited Enzyme
Substrate Analogues	
α-Fluoromethylglutamic acid	Glutamate Decarboxylase
α-Fluoromethylornithine	Ornithine Decarboxylase
α-FluoromethylDOPA	Aromatic Amino Acid Decarboxylase
α-Fluoromethyltyrosine	Aromatic Amino Acid Decarboxylase
α-Fluoromethylhistidine	Mammalian Histidine Decarboxylase
Product Analogues	
α-Fluoromethyldopamine	Aromatic Amino Acid Decarboxylase
α-Fluoromethylhistamine	Mammalian Histidine Decarboxylase
3-Fluoro-D-alanine	Bacterial Alanine Racemase

α-Fluoromethylamino acid



α-Fluoromethylamine



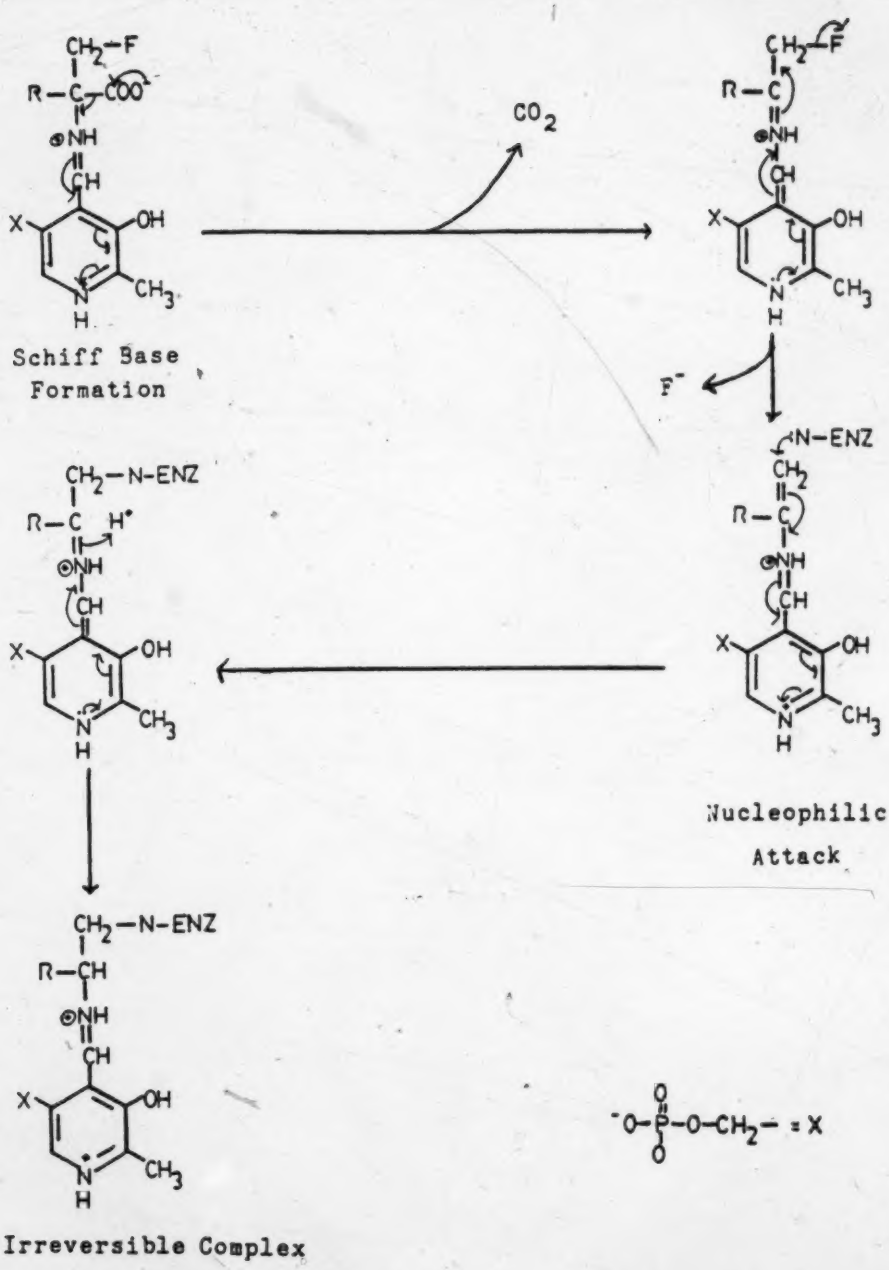


Figure I-5: Proposed method of enzyme inactivation by "suicide substrates" containing an α-fluoromethyl group (after Kollonitsch *et al.*[57]).

S-α-fluoromethyl-histidine has no effect on bacterial histidine decarboxylase as this enzyme does not employ pyridoxal phosphate as a cofactor. These irreversible inhibitors are stereospecific with the exception of α-fluoromethylhistamine where both enantiomers are weak histidine decarboxylase inhibitors. As well, the fluorinated enzyme substrate analogues tend to be more potent inhibitors than the fluorinated product analogues. The actual inhibiting activity is due to the 3-fluoroalanyl or α-fluoromethylamino group. This may explain why β-fluoro-histidine does not inhibit histidine decarboxylase[54] and why 4-amino-3-fluoro-butanoic acid is a GABA-T substrate and not an irreversible inhibitor.[72]

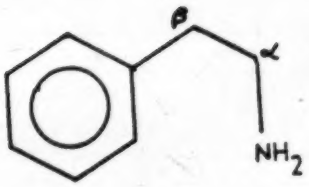
E. Effect of Aliphatic Fluorination of Sympathomimetic Amines

Nomenclature

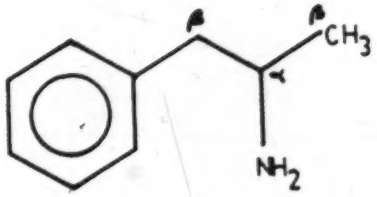
Fuller[26-36,60] in his work on fluorinated compounds uses the designation β to identify the site of fluorination in his compounds. In the case of the phenylethylamine (PEA) analogues this is an unambiguous nomenclature as only one carbon is β to the amino group (Fig. I-5). Unfortunately, in the case of the amphetamine analogues this designation can be confusing as there are two carbons β to the amino group (Fig. I-5). Thus, β,β-difluoroamphetamine (β,β-DFAM) could have either structure I or II shown in Figure I-6. Fuller's work has set the precedent of designating the benzylic carbon as the β carbon, therefore, in order to avoid confusion, amphetamine (AM) analogues fluorinated on the alternate β carbon (*i.e.* the terminal methyl carbon) will be referred to as α-fluoromethyl analogues of PEA.

Aliphatic β-Fluorination of Sympathomimetic Amines

The special physico-chemical properties of fluorine have also been applied to study the influence of ionization on the activity of sympathomimetic amines[26]. Employing the concepts of "isogeometric modification",[50] Fuller *et al.*[26-36] decided to use fluorine substitution to alter the pKa of chosen amines thereby altering the ratio of nonprotonated and protonated molecules



PEA



AM

I II

β,β-DFAM

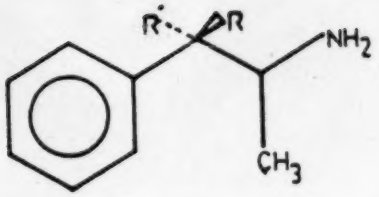
Figure I-6: Nomenclature of aliphatic-fluorinated sympathomimetic amines.

at physiological pH. Fluorination of the benzylic carbon was chosen since this is not a major metabolic or pharmacologic site in the AM series. Therefore, it was felt that any observed alterations in biochemistry or pharmacology would be due to altered degrees of protonation[26]. Many of the compounds studied by Fuller are the β,β-difluorinated analogues which have pKa's below physiological pH and therefore exist mainly as nonprotonated molecules under biological conditions. The compounds studied were: β,β-difluoroamphetamine, β,β-difluorophenylethylamine (β,β-DFPEA) and β,β-difluoro-p-chloroamphetamine (β,β-DFpCA) (Fig. I-7).

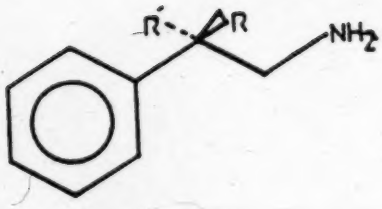
Amphetamine vs β,β-Difluoroamphetamine

Comparative studies between amphetamine (AM) and β,β-DFAM indicate that β,β-difluorination results in an analogue which is equipotent to AM with respect to some pharmacological properties but which is less potent in eliciting other effects. This is another example of how fluorination of a biologically active compound can separate multiple pharmacological effects. For example, at equimolar doses, AM and β,β-DFAM are equipotent at increasing serum free fatty acid levels in rats but only AM elicits a hyperthermic response[34]. However, at doses required to produce equivalent brain levels, the hyperthermic induction by AM and β,β-DFAM is similar[26]. This observation supports the view of Matsumoto[59] that AM-induced hyperthermia is not due to the mobilization of free fatty acids as had been proposed by Gessa *et al.*[37]. In this case, the separation of the multiple effects of AM is due to an alteration in distribution (*i.e.* decreased brain levels) rather than an alteration in the receptor-ligand interaction as observed with the fluorocorticoids and ring-fluorinated NA.

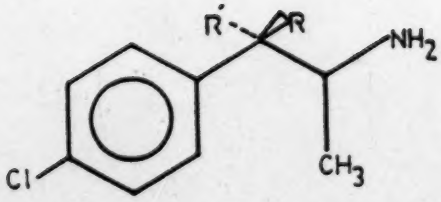
β,β-Difluorination also affects the metabolism of AM. In mammals AM is metabolized by two principal, mutually exclusive pathways: *para*-hydroxylation and oxidative deamination.[6,7] Rats metabolize AM almost entirely by *para*-hydroxylation (53 % of dose in 24 h) with very little deamination (3 % of dose), while mice both *para*-hydroxylate (14 % of dose) and deaminate (42 % of dose).[26] Although the half-lives of AM and β,β-DFAM in rat brain are similar, the two compounds are metabolized *via*



R	R'	Compound
H	H	AM
H	F	β-FAM
F	F	β,β-DFAM



H	H	PEA
H	F	β-FPEA
F	F	β,β-DFPEA



H	H	pCA
H	F	β-FpCA
F	F	β,β-DFpCA

Figure I-7: Structures of β-fluorinated sympathomimetic amines.

separate routes[26,28,29,31]. AM is mainly metabolized by *para*-hydroxylation while β,β-DFAM is metabolized through oxidative deamination[29]. This alteration in the preferred metabolic pathway may be due to a decreased electron density of the aromatic ring, thereby making it less amenable to *para*-hydroxylation or due to decreased electron density on the amino group (as supported by the decrease in pKa), possibly weakening the C-N bond to make deamination easier. Metabolism of β,β-DFAM *via* deamination is supported by the following observations:

1. pretreatment with desmethylimipramine (an inhibitor of *para*-hydroxylation) increases the AM levels in rat brain and increases the amount of AM excreted unchanged in urine but does not affect levels or excretion of β,β-DFAM[29];
2. pretreatment with phenobarbital (an inducer of hepatic microsomal enzymes) does not affect *in vivo* levels of AM in rat tissues but decreases levels of β,β-DFAM[31];
3. pretreatment with SKF-525 A and DPEA (inhibitors of hepatic microsomal enzymes) does not affect AM levels in rat brain but does increase levels of β,β-DFAM[29].

Metabolism also appears to be responsible for the observed differences in brain half-lives of AM and β,β-DFAM in mice, the half-life of β,β-DFAM being approximately one-third that of AM. As mice partly metabolize AM by deamination, it would appear that β,β-fluorination increases the rate of deamination resulting in faster metabolic clearance of β,β-DFAM compared to AM.[30]

Another difference between AM and β,β-DFAM is that AM depletes NA levels in rat brain and heart while β,β-DFAM leaves these levels unchanged from values in control animals. Fuller[26] felt the decrease in NA levels may be due to the *para*-hydroxylated metabolite. As β,β-DFAM does not *para*-hydroxylate, this may account for the observed difference.

Phenethylamine vs β,β-Difluorophenylethylamine

Comparative *in vitro* studies show that β,β-DFPEA is a better substrate for lung *N*-methyltransferase than PEA, but a poorer substrate for liver microsomal deaminase[26,31]. At equimolar doses, it appears that β,β-DFPEA is a poorer MAO

inhibitor than the monofluoro analogue. However when inhibition is correlated to the concentration of protonated species, the inhibition activities of the two analogues are equivalent.[26,60] Thus the protonated species appear to be responsible for MAO inhibition. As a substrate for MAO, β,β-DFPEA is not as good as PEA, so β,β-difluorination makes PEA more resistant to metabolism. This is in contrast to the increase in metabolism observed when AM is β,β-difluorinated. This may be due to the fact that AM is deaminated by microsomal enzymes while PEA is deaminated by mitochondrial MAO.[26,60]

Distribution of PEA is also affected by β,β-difluorination in a manner similar to that seen with AM (*cf.* β,β-DFAM). That is, after equimolar doses, the concentration of β,β-DFPEA found in fat is greater than that of PEA.[36] Even so, at equimolar doses β,β-DFPEA is more effective at inducing locomotor activity in mice than is PEA. This result seems to be opposite to the findings with AM and β,β-DFAM. In addition, at equimolar doses, PEA brain levels are lower than those of β,β-DFPEA. This too appears at first to be opposite to the observations with AM and β,β-DFAM. However, when mice are pretreated with tranylcypromine (an MAO inhibitor) the brain levels of PEA increase to a level higher than that of β,β-DFPEA. As a result, mice pretreated with tranylcypromine before treatment with PEA have higher locomotor activity levels than those mice treated with β,β-DFPEA after similar pretreatment. Thus when the rapid metabolism of PEA is inhibited, the effects of β,β-difluorination on PEA are similar to those observed with AM.[36] That is, the higher distribution of the β,β-difluoro analogues into fat and away from brain results in decreased central stimulant efficacy.

p-Chloroamphetamine vs β,β-Difluoro-p-chloroamphetamine

β,β-Difluorination of p-chloroamphetamine (pCA) to produce β,β-DFpCA results in an analogue with an decreased pKa and increased lipophilicity.[38] As with β,β-DFAM and β,β-DFPEA, β,β-DFpCA is localized to a larger extent into fat than is its parent compound.[26,35] In addition, the half-life of β,β-DFpCA in rat brain is one-quarter that of pCA. While *para*-chlorination blocks the metabolism of AM compounds by *para*-hydroxylation,[6,7] β,β-difluorination appears to shift the metabolism

of AM compounds toward deamination. Thus, while pCA is not as rapidly metabolized as AM due to the blockade of the major *para*-hydroxylation pathway, β,β-DFpCA is more rapidly metabolized than pCA.[26,35]

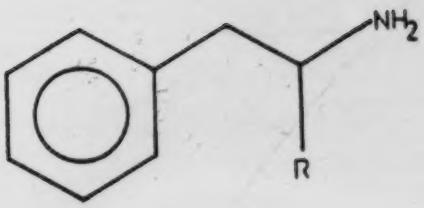
pCA causes rapid decreases in the vels of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), and these decreases may last several months.[26] When rats are treated with β,β-DFpCA at doses large enough to produce brain levels equimolar to pCA, it produces a decrease in 5-HT and 5-HIAA at 6 h that is similar to that observed after treatment with pCA. However, at 24 h after β,β-DFpCA treatment, 5-HT and 5-HIAA levels are back to normal while after pCA treatment these levels are still depressed.[35] The initial decrease in 5-HT relies on the active uptake of pCA and β,β-DFpCA into the 5-HT neurons.[27] Fuller[26] felt that the continuous uptake of pCA is responsible for the prolonged decreases of 5-HT and 5-HIAA. As β,β-DFpCA is rapidly removed from the brain by metabolism, it is not available for the required continuous uptake. In addition, it has been found[26] that β,β-DFpCA has a lower affinity (approximately one tenth) for the 5-HT reuptake system than does pCA. Hence the reduction of 5-HT and 5-HIAA by β,β-DFpCA is not as prolonged as that observed with pCA and the neurotoxic effect of pCA is lost.[32]

Aliphatic α-Fluoromethylation of Phenylethylamines

One of the less desirable characteristics of Fuller's compounds is that β-fluorination not only affects the pKa of the amino group but also perturbs the electron density of the aromatic ring to some extent. In order to study the effects of decreased pKa alone, Coutts *et al.*[12] synthesized α-fluoromethyl-β-phenylethylamine (FAM) and α-fluoromethyl-β-p-chlorophenylethylamine (FpCA) (Fig. 1-8).

α-Fluoromethyl-β-phenylethylamine

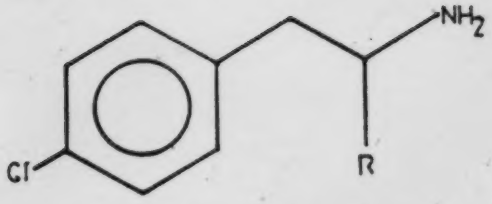
Fluoromethylation on the carbon α to the amino group of PEA alters the electron density of the nitrogen without affecting that of the aromatic ring. α-Fluoromethylation alters AM properties. For example, while (±)-FAM releases DA from rat striatum, it is



$R = CH_3$ AM

$R = CH_2F$ FAM

$R = CF_3$ TFAM



$R = CH_3$ pCA

$R = CH_2F$ FpCA

Figure I-8: Structures of α-fluoromethylated phenylethylamines.

not as potent as is (±)-AM[2,11]. Another, rather surprising difference is the change in MAO stereoselectivity observed with FAM. While only S-(+)-AM is a substrate for MAO-B, both enantiomers of FAM act as substrates, with the dextrorotamer being preferred[82]. Both optical isomers of FAM are also competitive inhibitors of MAO-B although they are not as potent as their respective AM enantiomers.

In rats, S-(+)-AM and R-(+)-FAM are equipotent at inducing hyperthermia but initial studies indicate R-(+)-FAM achieves higher brain levels than S-(+)-AM after equimolar doses[16,19]. In mice, the effect of R-(+)-FAM on body temperature is unlike that of S-(+)-AM. R-(+)-FAM elicits a biphasic temperature response, an initial transient hyperthermia followed by a prolonged hypothermia, while S-(+)-AM induces hyperthermia only. A similar biphasic effect is observed with locomotor activity in mice treated with R-(+)-FAM (*i.e.* increased activity followed by inactivity), while S-(+)-AM induces only hyperactivity[17].

α-Fluoromethyl-β-p-chlorophenylethylamine

Fluoromethylating p-chlorophenylethylamine on the carbon α to the amino group results in FpCA, a compound which is similar in its biochemical effects to β,β-DFpCA. *In vitro* studies indicate FpCA is not as potent as pCA at inducing 5-HT release from rat striatum.[2,11] In *in vivo* studies after equimolar doses, the brain level of FpCA in rats is lower than that of pCA, and FpCA disappears from the brain more rapidly than pCA. As well, at equimolar doses, FpCA is less potent than pCA at decreasing 5-HT levels, but at equivalent brain levels pCA and FpCA reduce 5-HT levels to the same extent at 1.5 h after treatment.[3,4] However, as with β,β-DFpCA, at 24 h after treatment with FpCA, 5-HT levels are back to normal. Thus, α-fluoromethylation inhibits the prolonged decrease in brain 5-HT levels. In addition, FpCA has been found to be a weaker inhibitor of 5-HT uptake than is pCA[3] as has been found for β,β-DFpCA.[26]

α-Trifluoromethyl-β-phenylethylamine

Pinder *et al.* synthesized the trifluoromethyl analogue of PEA,[66] α-trifluoromethyl-β-phenylethylamine (TFAM) (Fig. I-8), to study the effect of fluorination on the pharmacological effects of AM.[65,66] TFAM is without AM-like activity, *i.e.* it does not reverse reserpine sedation in mice, has no anorectic activity in rats or dogs nor does it deplete catecholamines in rat heart or brain. Pinder[65] felt that the lack of activity is due either to the decreased ability of TFAM to cross the blood-brain barrier or to the "severe reduction" in the availability of the nitrogen lone pair as illustrated by the very low pKa of TFAM compared to AM (4.97 *vs* 9.93, respectively).[65]

F. Behavioral and Pharmacological Effects of AM

The purpose of this thesis is to determine how behavioral (locomotor activity) and pharmacologic (body temperature) effects relate to brain levels of the optical isomers of AM and FAM. As this thesis will compare FAM to AM, it is necessary to review the behavioral and pharmacological effects of AM.

Although it possesses a relatively simple chemical structure, AM has a wide spectrum of pharmacological, biochemical and behavioral activities. Central nervous stimulation is the main pharmacological action of AM.[13,39,46] This central nervous stimulation is responsible for several AM effects such as anorexia, insomnia, stereotypy, increased basal body temperature and increased locomotor activity.[46]

A commonly studied behavioral effect of AM is the induction of locomotor activity. The (+) enantiomer is a more potent inducer of locomotor activity than is the (-) enantiomer. The maximum locomotor increase induced by S-(+)-AM in rats occurs at approximately 10 μmol/kg; at doses of 35 to 75 μmol/kg the locomotor stimulation is replaced by stereotypyes.[46] A stereotypy is defined as a "persistent repetition of ... movement without meaning".[80] In rats and mice these stereotypyes induced by AM include: sniffing, grooming (possibly accompanied by salivation), head movements, rearing and gnawing.[46] These stereotypyes are dose-related in that at low doses (≈10 μmol/kg) the main behavioral effect

observed is increased locomotor activity, while at intermediate doses (≈35 μmol/kg) locomotor activity is replaced by grooming, rearing and sniffing. As the dose is increased the number of separate stereotypyes observed decreases until at high doses (≈70 μmol/kg) only gnawing is seen, which may be expressed as self-mutilation. In mice, the gnawing may be accompanied by backwards locomotion, depending on the size of the test environment. The (+) enantiomer is "only slightly" more potent than the (-) enantiomer at inducing stereotypyes.[46]

Most of the effects of AM are thought to be due to its ability to increase the release of newly synthesized NA and DA from neurons.[13,39,46] Thus α-methyltyrosine, a tyrosine hydroxylase inhibitor which prevents the synthesis of NA and DA, blocks the central stimulant effects of AM. However AM is capable of reversing reserpine-induced sedation in mice as reserpine causes depletion of stored NA and DA but does not affect catecholamine synthesis. The central stimulant effects of AM may be blocked by pretreatment with haloperidol (a DA receptor blocker) and by selectively destroying DA pathways with 6-hydroxydopamine (a catecholamine neurotoxin). However interference with noradrenergic pathways by α- or β-adrenoceptor blockers, or by treatment with disulfuram (a dopamine-β-hydroxylase inhibitor) to prevent NA synthesis do not inhibit these AM effects. So, although AM induces the release of both NA and DA, only DA release is responsible for the stereotypyes and the increases in locomotor activity and basal body temperature.[9,46]

AM is also an inhibitor of mitochondrial MAO and blocks catecholamine uptake. While the latter two effects are not likely to be the main reason for observed AM activities, they may potentiate the central stimulant effects.

II. Materials and Methods

A. Chemicals

Unless otherwise specified, all chemicals and organic solvents were obtained from Fisher Scientific Ltd.. Toluene and ethyl acetate were purchased from B.D.H. Chemicals while isopentane was obtained from Aldrich Chemical Co.. The derivitizing agent, pentafluorobenzoyl chloride (PFBC) was obtained from Aldrich Chemical Co. and stored at 0°. The hydrochloride salts of S-(-) and R(+)-FAM were synthesized from the corresponding isomers of phenylalanine according to a method based on that of Coutts *et al.*.[12] The sulfate salts of S(+)- and R(-)-AM were obtained from Health and Welfare Canada. Sodium phenobarbital was obtained from Smith, Kline and French. Organic solvents were glass-distilled prior to use in analyses. A Milli-Q reagent water system (Millipore) was used to provide double-distilled water for analytical use. Gases used in the analyses were obtained from either Liquid Carbonic or Linde (Union Carbide).

B. Animals

Male mice of the Balb/cCr strain or female mice of the Balb/cCr or ICR strain (body weight 18-27 g) or male Sprague-Dawley rats (body weight 180 - 250 g) were used in all experiments. Unless otherwise specified, animals were allowed free access to food (Purina Rat Chow®) and water and were housed in plexiglass cage boxes with stainless steel barred lids (rats) or stainless steel mesh lids (mice).

Injection Solutions

All drugs were dissolved in saline (0.9% w/v) and injected i.p. at a dose volume of 10 mL/kg for mice and 1 mL/kg for rats. All doses were calculated on the basis of free base (amines) or free acid (phenobarbital).

C. Apparatus and Instrumentation

Open Field Activity Box

Locomotor activity (open field activity) was monitored in a square field (60 X 60 cm) divided into a grid of 9 squares, each square measured 20 X 20 cm. At the beginning of each reading, the animal was moved into the central square of the grid and activity was measured as the number of squares entered by all four feet during a 3 min period.

Rectal Thermometer

Rectal temperatures were read 30 sec after insertion of a small animal probe (Yellow Springs Instruments, Model 43TA, 400 series probe) to a depth of 2.5 cm for mice and 4 cm for rats. Room temperature was maintained between 22 and 27° during all experiments.

Balances

Analytical Balance

Compounds for the preparation of analytical standard solutions or drug injection solutions and tissue samples were weighed on a Mettler analytical balance (Model H10). All tissue samples and compounds were weighed to within 0.1 mg.

Top-Loading Balance

All animals were weighed on a Sartorius top-loading balance. Animals were weighed to 0.1 g.

Tissue Homogenizer

Homogenization of brain and fat samples in ice-cold perchloric acid (0.4 N) was performed using an homogenizer from Eberbach Corporation equipped with an A.C. motor (115 V, 0.7 amp, Bodine Electric Company), a Teflon®-stainless steel pestle and a glass mortar.

Centrifuges

Microfuge

Plasma was separated from blood samples using a Fisher Micro-Centrifuge Model 235B.

Bench Centrifuge

A Dynac multiple speed centrifuge (Clay Adams, division of Becton, Dickinson and Company) was used to separate solvent phases during analyses. The centrifuge had variable speed settings from 0 to 100 and in order to separate solvent phases a setting of 100 was used for 10-15 min.

Refrigerated Centrifuge

All brain and fat homogenates were centrifuged in a Damon/IEC Model B-20A refrigerated centrifuge. Homogenates were centrifuged at 10,000 X g for 10 min at 4°.

Vortex Mixer

Samples were mixed on an IKA-Vibrax-VXR multiple vortex mixer (Janke and Kunkle GmbH, IKA-Werk) capable of handling 36 samples.

Gas-Liquid Chromatography

Gas-liquid chromatography was performed using a Perkin-Elmer Sigma 3 Gas Chromatograph (GC) equipped with an electron capture detector (ECD) with a ^{63}Ni electron source. Chromatography was performed using bonded-phase fused silica columns (0.32 mm ID, 8-10 m in length, J & W Scientific Inc.) with a DB-5 stationary phase (equivalent to SE-54). Equivalent separations were obtained on columns with stationary phase thicknesses of either 1 μm or 0.25 μm provided that the chromatographic conditions were set appropriately as described below:

1. For the 1 μm coated column, the GC oven temperature was maintained at 100° for 1 min

and then increased to 250° at a rate of 6° per min;

2. For the 0.25 μm coated column, the GC oven temperature started at 100° and was immediately increased to 250° at a rate of 4° per min.

Otherwise, the GC operating conditions were the same for both columns. Helium carrier gas was delivered to give a column flow rate of 1-3 mL/min. Argon/methane (95:5) make-up gas was delivered to result in a total gas flow rate of 60mL/min at the ECD outlet. The injection port temperature was 300° while the ECD was maintained at 350°.

Mass Spectrometry

Electron impact and chemical ionization mass spectrometry were performed by personnel of the Department of Chemistry (University of Alberta), on a VG 70-E double focusing magnetic sector mass spectrometer (Vacuum Generators). Sample introduction was by a GC (Varian Vista 6000).equipped with a 30 m DB-1 fused silica column, the injection port was heated to 250° and the column was initially heated to 100° and immediately increased to 290° at 10° per min. The mass spectrometer conditions were: ion source temperature, 200°; ionization voltage, 70 eV; accelerating voltage, 6000 V. Total ion chromatographs indicated baseline resolution of the PFBC derivatives of AM, FAM and pCA.

D. Pharmacological and Behavioral Methods

Effects of FAM and AM: Mice

Individual male Balb/cCr mice were placed into the open field box. After 1 h, activity was measured and immediately thereafter the animal was treated i.p. with saline, R-(+)- or S-(-)-FAM (6.5, 16, 33 or 65 μmol/kg) or S-(+)- or R-(-)-AM (16, 33 or 65 μmol/kg). Activity was measured again at 30, 60, 90, 120, 180, 240 and 480 min after treatment.

The effect of each enantiomer of FAM or AM on rectal temperature was determined in male Balb/cCr mice. Rectal temperatures were taken just prior to treatment with saline or an enantiomer of FAM or AM (16, 33 or 65 μmol/kg). Rectal temperatures were taken again 30,

60, 90, 120, 180, 240 and 480 min after injection.

The effect of gender on the thermic response to the enantiomers of FAM was also examined. The rectal temperatures of male or female mice of the Balb/cCr strain were determined just prior to the injection of saline or R-(+)- or S-(-)-FAM (65 μmol/kg) and again at 30, 60, 90, 120, 180, 240 and 480 min after injection.

The dependence of the locomotor response upon the dose of R-(+)- or S-(-)-FAM was determined by treating male Balb/cCr mice with saline or R-(+)- or S-(-)-FAM (6.5, 16, 33 or 65 μmol/kg) and measuring locomotor activity 180 min after injection.

The dose-dependence of the temperature response was determined similarly on other male Balb/cCr mice except that at 180 min after treatment rectal temperatures were recorded.

Effect of Enzyme Induction on FAM-Induced Changes in Rectal Temperature: Rats

Rats were pretreated with phenobarbital (i.p., 40 mg/kg) twice a day (8 and 17 h) for four days. On the fifth day, instead of receiving phenobarbital at 8 h, the rats had their rectal temperatures taken just prior to injection of 25 μmol/kg of R-(+)-FAM or S-(+)-AM. Rectal temperatures were measured 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330 and 360 min after treatment.

E. Tissue Distribution of AM and FAM

Brain Levels of FAM and AM: Mice

Male or female mice of the Balb/cCr strain were injected i.p. with either of the optical isomers of FAM (65 μmol/kg) and were sacrificed by cervical dislocation at 30, 60, 90, 120, 150 or 180 min after injection. Brains were rapidly removed, blotted dry and frozen in isopentane and dry ice. After freezing, brains were blotted dry, weighed and homogenized in ten volumes of ice-cold perchloric acid (0.4N) and were immediately assayed as described below. Other male Balb/cCr mice were injected with either isomer of AM (65 μmol/kg) and processed in an identical manner except sacrifices took place 30, 60, 120 or 180 min after injection.

Distribution of S-(-)-FAM in Brain, Fat and Plasma

Male mice of the Balb/cCr strain or female mice of the ICR strain were injected with 65 μmol/kg of S-(-)-FAM and sacrificed at 5, 15, 30, 60, 90 or 120 min after injection. Trunk blood was collected over solid sodium citrate (10 - 20 mg), mixed and immediately centrifuged (12,000 X g, 1 min) to obtain a plasma sample. The volume of the plasma obtained was determined using a micro-pipet. Brains were collected and treated as for brain tissue above. Omental fat was collected and homogenized in 10 volumes of ice-cold perchloric acid (0.4 N) and immediately assayed as described below.

Analysis of FAM or AM in Brain, Fat and Plasma: Mice

Tissue levels of FAM or AM were determined according to a method based on that of Cristofoli *et al.*[15] The internal standard, p-chloroamphetamine (pCA), was added (300 ng for FAM analysis, 2000 ng for AM analysis) to plasma (20-100 μL) or to the perchloric acid homogenates of brain or fat (5-10 mL). Plasma was then diluted with an equal volume of perchloric acid. Tissue homogenates and diluted plasma were centrifuged in a refrigerated centrifuge (4°, 10,000 X g, 10 min) and supernatants were removed, basified by additon of solid sodium carbonate (pH~9.5) and extracted with chloroform (2 X 5 mL for tissues, 2 X 1 mL for plasma) by shaking for 20 min on a multiple sample vortex mixer. Samples were centrifuged to separate the phases. The combined chloroform layers were extracted with 1.2 N hydrochloric acid (2 mL). The acid phase was collected and basified with solid potassium carbonate. Then PFBC (5 μL) was added along with 3 mL of ethyl acetate. The reaction mixture was shaken for 20 min and centrifuged to separate the phases. The ethyl acetate phase was removed and evaporated to dryness under a stream of nitrogen at 30-38°. The resulting residue was dissolved in 100 μL of toluene and the solution was washed with ammonium hydroxide (0.7N, 200 μL). One microlitre portions of the toluene phase were injected into a GC (splitless injection) containing a DB-5 bonded phase silica capillary column with a 1 μm coating.

Compounds were quantitated by comparison of peak height ratios (compound of interest/internal standard) to standard curves constructed by plotting peak height ratios *vs*

amount of compound of interest, obtained by analysing homogenates of the appropriate tissue spiked with known amounts of drug and internal standard. Standard curves were linear over the range of 0.2-30.0 ng (on column) for both AM and FAM.

Brain Levels of R-(+)-FAM and S-(+)-AM: Rats

Rats were injected with R-(+)-FAM (10, 25 or 75 μmol/kg) or S-(+)-AM (25 μmol/kg) and were sacrificed at 1, 2 or 4 h after injection. Brains were removed and treated as for mouse brain except 20 mL of perchloric acid was used to homogenize the tissue.

Analysis of AM or FAM in Rat Brain

The method used was the same as that used for mouse brain except:

1. 2000 ng of internal standard (pCA) were added
2. 2 X 10 mL of chloroform were used for extraction, combined volumes were evaporated to ~10 mL
3. 1 mL of toluene and 2 mL of 0.7N ammonium hydroxide were used
4. the GC column used was a DB-5 (10 m) with a 0.25 μm coating

F. Urinary Excretion

Urine Collection: Mice

Immediately after injection of 65 μmol/kg of R-(+)or S-(-)-FAM or S-(+)- or R-(-)-AM, male mice of the Balb/cCr strain were placed singly into a 500 mL beaker equipped with a wire mesh platform. Animals were deprived of food during the collection but had free access to sugar water (6% sucrose w/v). Urine was collected for 24 h and the entire volume from each collection was assayed for AM or FAM.

Urine Collection: Rats

Immediately after injection of 65 μmol/kg of R-(+)or S-(-)-FAM or S-(+)- or R-(-)-AM, rats were placed singly into polypropylene metabolism cages. Animals had free access to food and water during the collection. Urine was collected 4, 8 and 24 h after injection, the entire volume of each collection was assayed for AM or FAM.

Analysis of AM and FAM in Mouse and Rat Urine

Internal standard (pCA) was added to each sample (5 μg for FAM analysis, 20 μg for AM analysis). The sample was acidified with 0.4N perchloric acid and centrifuged in a refrigerated centrifuge. The supernatant was analysed according to the method for analysis of FAM and AM in brain, fat and plasma except that the DB-5 column used had a 0.25 μm coating.

G. Determination of Partition Coefficients

Equal volumes of octanol and either pH 7.4 buffer [1] or 0.1 N NaOH were shaken in a one litre separatory funnel to saturate the phases and then allowed to separate. These phases were used throughout the determination. The solutions used in this experiment were 100 μg/mL free base of R-(+)-FAM or S-(+)-AM in octanol saturated 0.1 N sodium hydroxide or in octanol saturated isotonic phosphate buffer (pH 7.4).

In a 15 mL screw top pyrex culture tube, 5 mL of aqueous test solution, and a volume of octanol (2 mL for 0.1 N NaOH and 5 mL for pH 7.4 buffer) were mixed on a vortex mixer for 20 min. The tubes were allowed to stand 20 min to separate the phases. Then an aliquot was taken from each phase (1 mL aqueous or 0.2 mL octanol). The octanol aliquot was extracted with 1 mL of 1.2 N hydrochloric acid. The acid extract and the aqueous aliquot were basified with solid potassium carbonate then reacted with PFBC and analysed as for AM and FAM in rat brain tissue.

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[1] isotonic pH 7.4 buffer: 8.1 g anhydrous sodium phosphate dibasic, 1.8 g sodium phosphate monobasic monohydrate and 3.9 g sodium chloride all dissolved in distilled, demineralized water and diluted to one litre.

H. Statistical Analysis

Data are presented as mean ± S.E.M.. Differences between sets of data were determined by a two-tailed Student's *t*-test. Half lives of FAM or AM were determined by linear regression after application of appropriate curve feathering techniques.[38] Differences between slopes of plots were determined by comparison of the linear regressions followed by a two-tailed Student's *t*-test.[77]

III. Results

A. Behavioral and Pharmacological Results

Locomotor Activity in Mice

Male Balb/cCr mice treated with R-(+)-FAM showed a biphasic change in open field activity (Fig. III-1). Although mice treated with 6.5 μmol/kg of R-(+)-FAM were similar to saline-treated mice at all time points, mice which received 16 μmol/kg of R-(+)-FAM showed significantly ($p<0.05$) reduced locomotor activity compared to controls at 90, 120, 180 and 240 min after i.p. injection. As the dose was increased, the depression of locomotor activity was preceeded by a delay of onset at 33 μmol/kg and by significantly ($p<0.05$) increased locomotor activity (30 min after injection) at 65 μmol/kg. Grooming was observed in two of eight mice and increased sniffing was noted in all of the animals during the period of locomotor stimulation. S-(-)-FAM also significantly ($p<0.05$) reduced the locomotor activity at the 16, 33 or 65 μmol/kg levels, but did not induce increased locomotor activity at any dose tested nor was any stereotypic behavior observed in mice treated with S-(-)-FAM. The enantiomers were similar in their ability to produce prolonged, dose-dependent (Fig. III-2) reductions in locomotor activity. During the periods of decreased locomotor activity, mice appeared to be lightly sedated but were easily aroused by a handclap or whistle.

Male Balb/cCr mice treated with 16 or 33 μmol/kg of S-(+)-AM exhibited increased locomotor activity (Fig. III-3). Mice treated with 65 μmol/kg of S-(+)-AM exhibited decreased locomotor activity as well, however this decrease was associated with a high level of stereotypy, that is chewing and backwards locomotion and in the case of two animals (out of eight) self mutilation.

Mice treated with 16 μmol/kg of R-(-)-AM were similar to saline mice. Mice injected with higher doses of R-(-)-AM did show brief periods of decreased locomotor activity (at 30 and 60 min for 33 μmol/kg and at 60 and 90 min for 65 μmol/kg). These periods of decreased locomotor activity were associated with stereotypic behavior consisting of sniffing (33 or 65

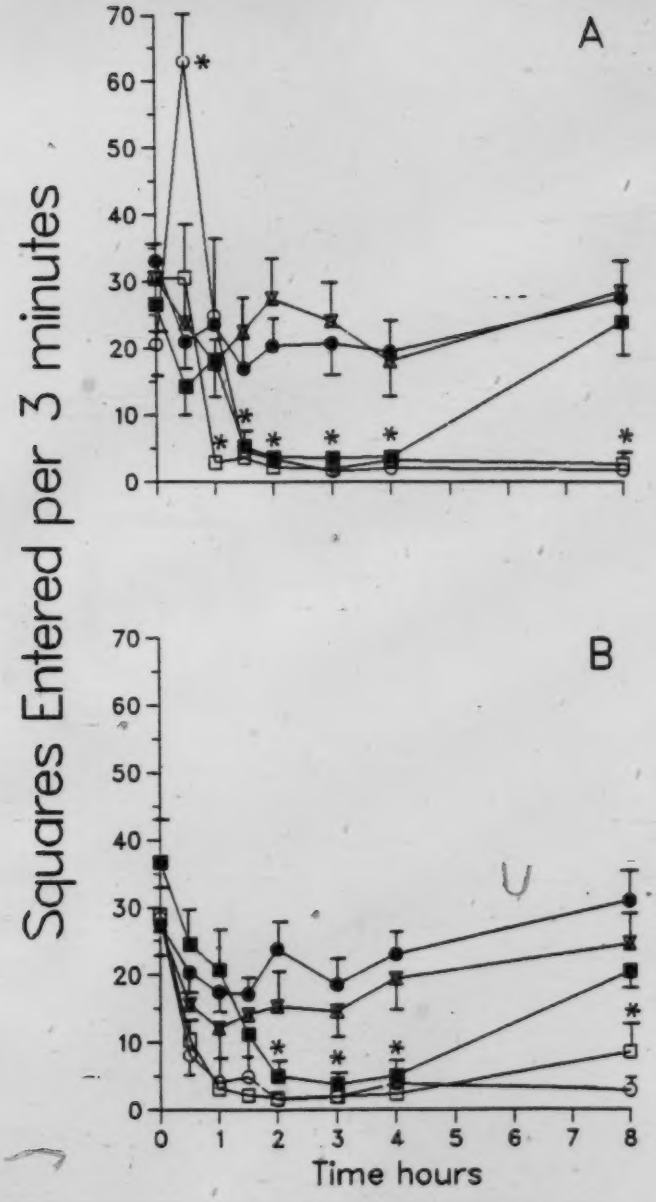

Figure III-1: Effect of R-(+)-FAM (A) and S-(-)-FAM (B) on locomotor activity in male Balb/cCr mice (n=8, mean quadrants entered ± S.E.M., except where S.E.M. < symbol size. • saline; ▣ 6.5 μmol/kg; ■ 16 μmol/kg; □ 33 μmol/kg; o 65 μmol/kg; * lowest dose which produces a significant difference ($p<0.05$) from saline).

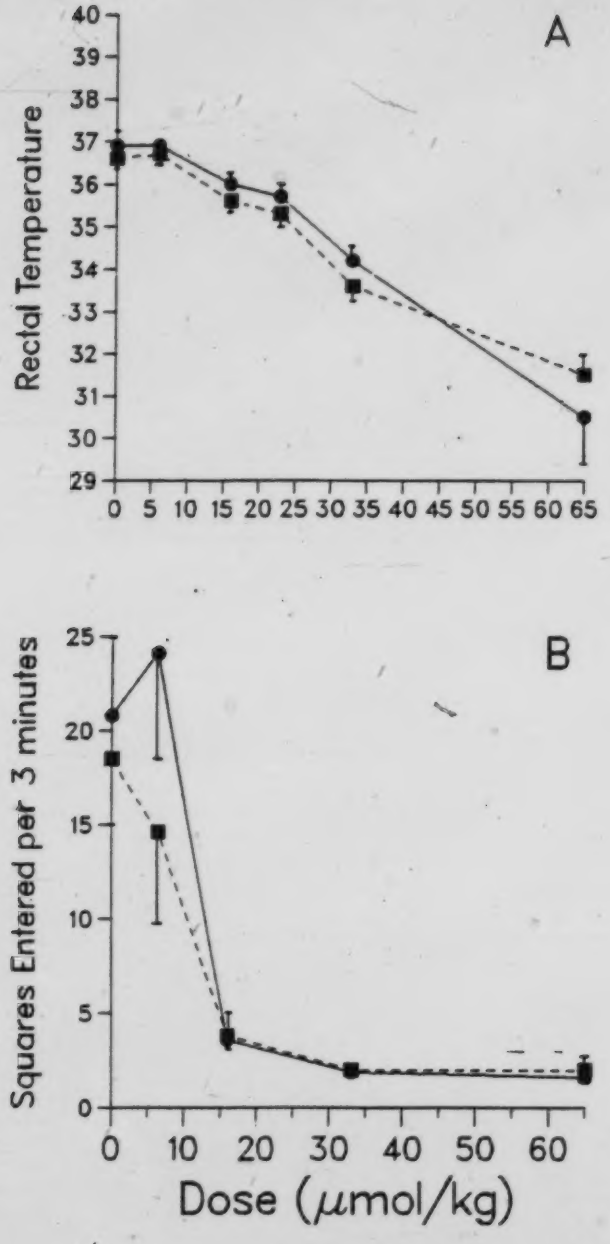

Figure III-2: Dose-dependent reduction in rectal temperature (A) and locomotor activity (B) in male Balb/cCr mice after R-(+)-FAM (•) and S-(-)-FAM (■) (n=5, mean rectal temperature ± S.E.M; n=8, mean quadrants entered ± S,E.M.; measurements taken 180 min after dosing).

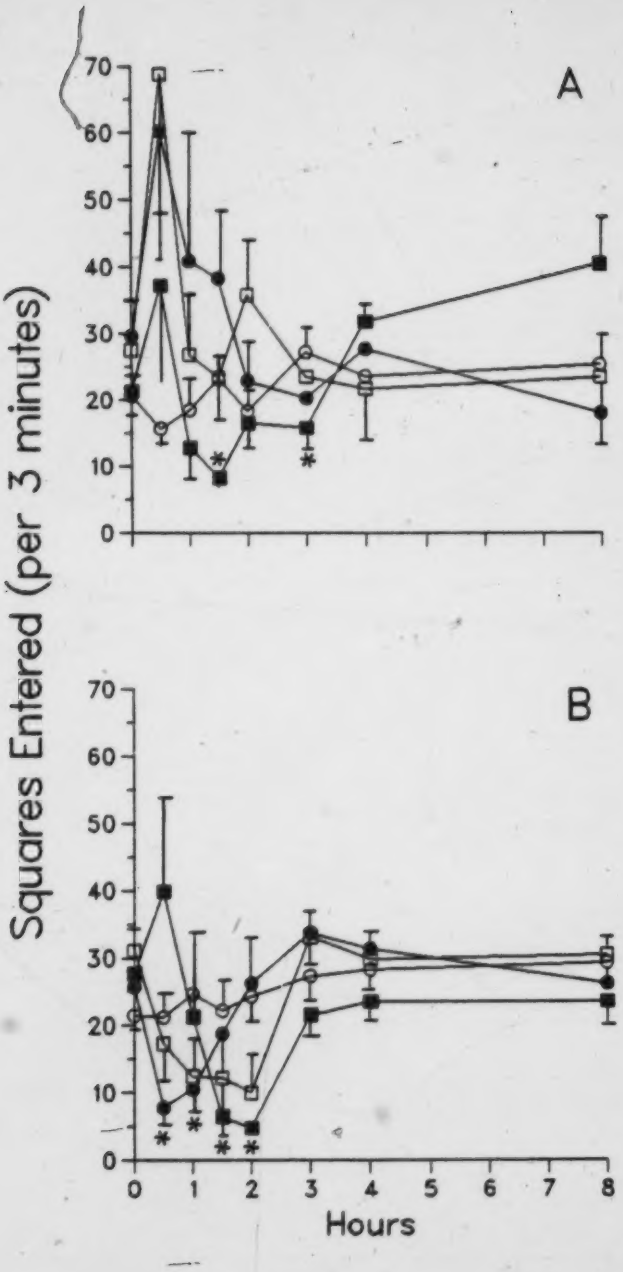

Figure III-3: Effect of S-(+)-AM (A) and R-(-)-AM (B) on locomotor activity in male Balb/cCr mice (n=8, mean quadrants entered ± S.E.M., except where S.E.M. < symbol size, o saline; • 16 μmol/kg; □ 33 μmol/kg; ■ 65 μmol/kg; * lowest dose which produces a significant difference ($p<0.05$) from saline).

μmol/kg) and grooming (65 μmol/kg) but did not develop into the high level stereotypyes of gnawing and backwards locomotion.[46]

After 24 h, all mice appeared to be normal and not different from saline-treated mice.

Rectal Temperatures in Mice

Both enantiomers of FAM caused a prolonged decrease in the rectal temperatures of male Balb/cCr mice. This decrease was dose-dependent (Fig. III-2) and followed a time course (Fig III-4) similar to that of the decrease in locomotor activity. R-(+)-FAM appeared to have a slight delay in the onset of hypothermia, which may have been due to the trend toward a transient increase in rectal temperature displayed by some of the mice treated with higher doses of this compound. However, the magnitude of the hypothermia was similar for both enantiomers with the maximum decrease at 3 h.

Female mice treated with 65 μmol/kg of either enantiomer of FAM showed no significant difference from males in magnitude or duration of thermic response except at 60 min after treatment with R-(+)-FAM when temperatures of female mice were significantly ($p<0.05$) higher than those of male mice (Table III-1).

At doses of 33 or 65 μmol/kg, the S-(+) enantiomer of AM caused a significant ($p<0.05$) increase in rectal temperature at 30 min (Fig. III-5). The enantiomers of AM both produced significant ($p<0.05$) but transient decreases in rectal temperature (Fig. III-5). These decreases were neither as great (eg. -2° decrease for S-(+)-AM *vs* -7.5° decrease for R-(+)-FAM) nor as prolonged (1 h for the AM enantiomers *vs* 8 h or more for the FAM enantiomers) as those produced by the FAM enantiomers and also were not clearly dose-dependent.

Rectal Temperatures in Rats

Rats treated with 25 μmol/kg of S-(+)-AM or R-(+)-FAM exhibited similar increases in rectal temperatures (Figure III-6) as was previously reported by Danielson *et al.*[16] However, after treatment with phenobarbital in order to induce microsomal enzymes, the

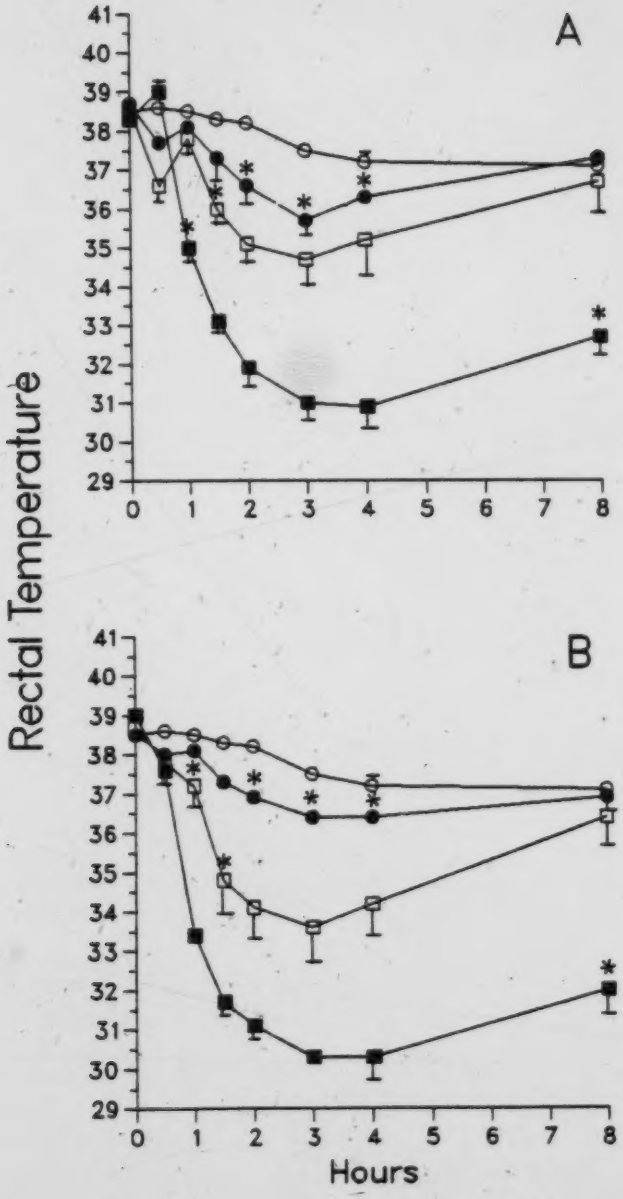

Figure III-4: Effect of R-(+)-FAM (A) and S-(-)-FAM (B) on rectal temperatures in male Balb/cCr mice (n=6, mean temperature ± S.E.M., except where S.E.M. < symbol size, o saline; • 16 μmol/kg; □ 33 μmol/kg; ■ 65 μmol/kg; * lowest dose which produces a significant difference ($p<0.05$) from saline).

Table III-1: Comparison of rectal temperatures in male and female Balb/cCr mice after treatment with R-(+)-FAM and S-(-)-FAM (65 μmol/kg, mean rectal temperature ± S.E.M.).

	0	30	60	90	120	180	240	480
Saline								
Female	38.3	38.6	38.9	38.6	38.7	38.3	38.2	37.6
(n=6)	± 0.2	± 0.1	± 0.1	± 0.1	± 0.2	± 0.2	± 0.4	± 0.4
Male	38.1	38.5	38.2	38.0	37.9	37.4	36.6	36.2
(n=6)	± 0.4	± 0.1	± 0.4	± 0.5	± 0.5	± 0.6	± 0.8	± 1.4
R-(+)-FAM								
Female	38.8	38.6	35.5*†	33.1*	32.7*	31.3*	31.3*	34.7*
(n=7)	± 0.1	± 0.4	± 0.4	± 0.3	± 0.6	± 0.6	± 0.7	± 0.8
Male	38.3	38.4	34.4*	32.7*	31.7*	31.1*	31.4*	33.8
(n=8)	± 0.2	± 0.3	± 0.2	± 0.3	± 0.4	± 0.6	± 0.7	± 1.2
S-(-)-FAM								
Female	39.0	37.0*	33.7*	32.1*	31.4*	31.0*	31.3*	33.3*
(n=6)	± 0.3	± 0.4	± 0.3	± 0.2	± 0.4	± 0.5	± 0.4	± 0.5
Male	39.1	37.4	33.1*	31.5*	31.2*	30.6*	30.6*	33.1
(n=6)	± 0.3	± 0.5	± 0.3	± 0.3	± 0.4	± 0.3	± 0.8	± 0.5

* significant ($p<0.05$) decrease from saline-treated mice of the same sex

† significant ($p<0.05$) difference from similarly treated male mice

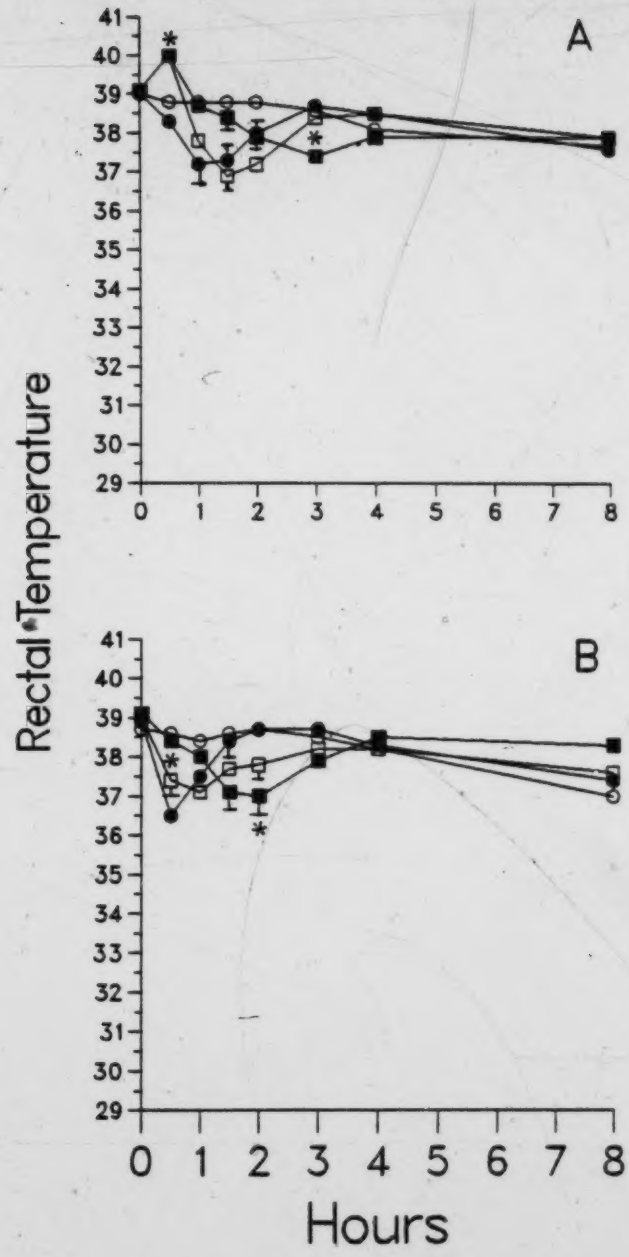

Figure III-5: Effect of S-(+)-AM (A) and R-(-)-AM (B) on rectal temperatures in male Balb/cCr mice (n=6, mean temperature ± S.E.M., except where S.E.M. < symbol size, o saline; • 16 μmol/kg; □ 33 μmol/kg; ■ 65 μmol/kg; * lowest dose which produces a significant difference ($p<0.05$) from saline).

hyperthermic response to R-(+)-FAM was reduced whereas that to S-(+)-AM was not modified (Figure III-6).

B. Analysis of AM and FAM

Analytical Method

Recoveries of FAM, AM and pCA from brain tissue were between 78 and 88% (Table III-2). Ratios of FAM or AM to pCA were similar after extraction from brain and derivitization and after derivitization alone. Slopes of the standard curves (Fig. III-7,8) were reproducible. Daily FAM/pCA and AM/pCA ratios were also reproducible. The limits of detection of FAM and AM were defined as twice the "control concentration", that is twice the background found in the brains of untreated or saline-treated mice. Traces of control and treated mice are shown in Figures III-9,10. The identities of the peaks of interest were confirmed by GC/MS (Fig. III-11,12,13).

Brain Levels of AM and FAM in Mice and Rats

The data obtained from male Balb/cCr mice treated with equimolar doses of AM or FAM indicate that the kinetics differ between the drugs (Fig. III-14). There was no significant difference between the brain levels achieved by S-(+)- and R-(-)-AM at any time, nor were the slopes of the log brain levels *vs* time curves, and therefore the half-lives, significantly different. The half-lives calculated for S-(+)-AM and R-(-)-AM were 42 and 48 min respectively (Table III-3). However, there was a significant ($p<0.05$) difference between the brain levels of the enantiomers of AM and those of the corresponding enantiomers of FAM, and, at all times, brain levels of FAM were lower than those of AM. In addition, while the brain levels of AM declined in a log-linear manner, the brain levels of each enantiomer of FAM declined in a biphasic manner consisting of an initial rapid decrease (between 30 and 90 min) followed by a slower decrease. The half-lives of each elimination phase of each enantiomer of AM and FAM are given in Table III-3. In male mice, brain levels

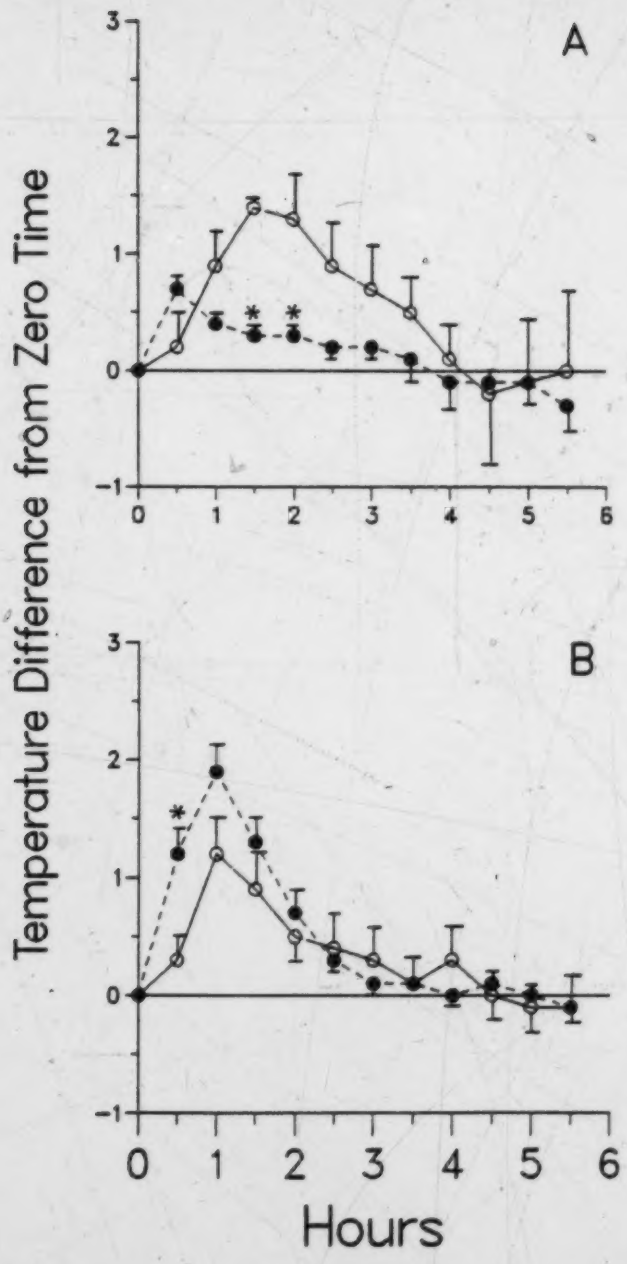

Figure III-6: Effect of R-(+)-FAM (A) and S-(+)-AM (B) (25 μmol/kg) on the rectal temperature of rats pretreated with saline (o) or phenobarbital (•, 100 mg/kg) (n=6, mean rectal temperature ± S.E.M.; * significant difference ($p<0.05$) from saline-treated rats).

Table III-2: Analytical statistics of the method used to determine AM and FAM in brain tissue (mean ± S.E.M.).

	AM	FAM	pCA
% Recovery:	82.5 ± 2.2 (n=5)	87.9 ± 1.5 (n=5)	78.6 ± 2.2 (n=10)
Ratios:			
Brain Extraction (5 ng)	1.03 ± 0.06 (n=5)	0.57 ± 0.01 (n=5)	—
Derivitization only (5 ng)	1.03 ± 0.03 (n=6)	0.56 ± 0.01 (n=6)	—
Daily Ratios*:			
1 ng	—	0.17 ± 0.02 (n=7)	—
10 ng	0.57 ± 0.09 (n=4)	1.58 ± 0.06 (n=6)	—
30 ng	2.20 ± 0.19 (n=4)	—	—
Standard Curves:			
Slopes:			
High Level (2.0-30 ng)	0.88 ± 0.08 (n=4)	1.56 ± 0.08 (n=9)	—
Low Level (0.2-2.0 ng)	—	1.22 ± 0.11 (n=11)	—
Intercepts:			
High Level	-0.1 ± 0.2 (n=4)	0.00 ± 0.03 (n=9)	—
Low Level	—	0.07 ± 0.03 (n=11)	—
Coefficient of Correlation (r^2):			
High Level	0.9876 ± 0.0051 (n=4)	0.9889 ± .0032 (n=9)	—
Low Level	—	0.9905 ± 0.0019 (n=11)	—
Limits of Detection:			
(ng)	0.50 ± 0.09 (n=4)	0.20 ± 0.05 (n=11)	—

* AM/pCA or FAM/pCA

NB: all weights given are on column amounts

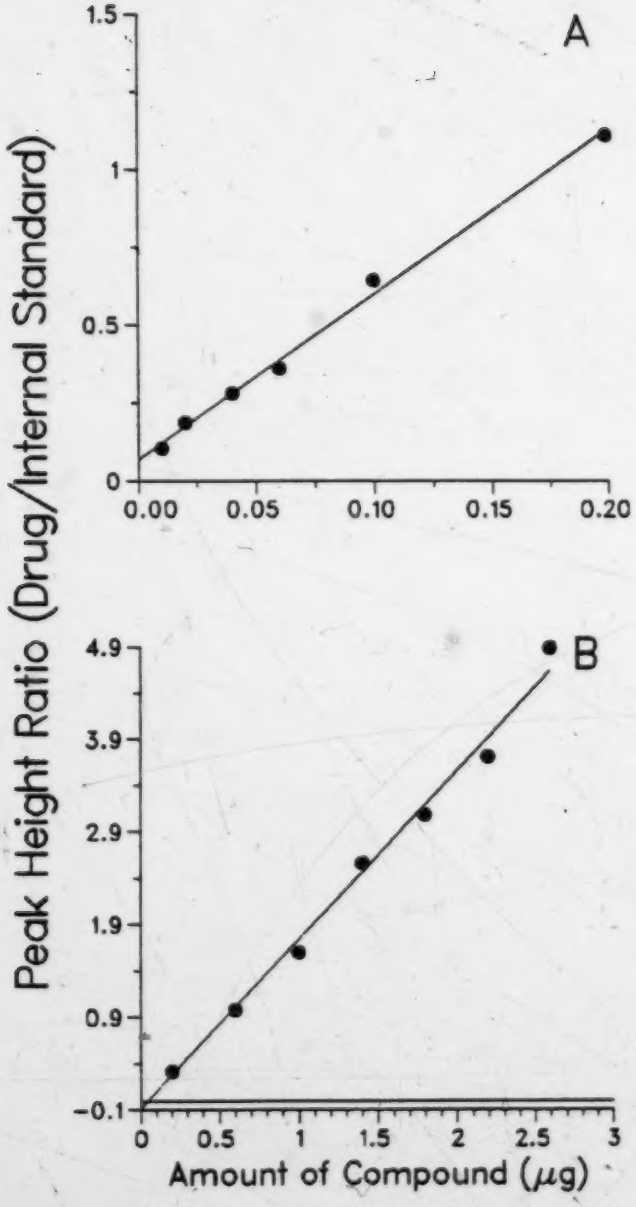

Figure III-7: Standard curves of FAM (low level (A) and high level (B)) in brain tissue homogenates.

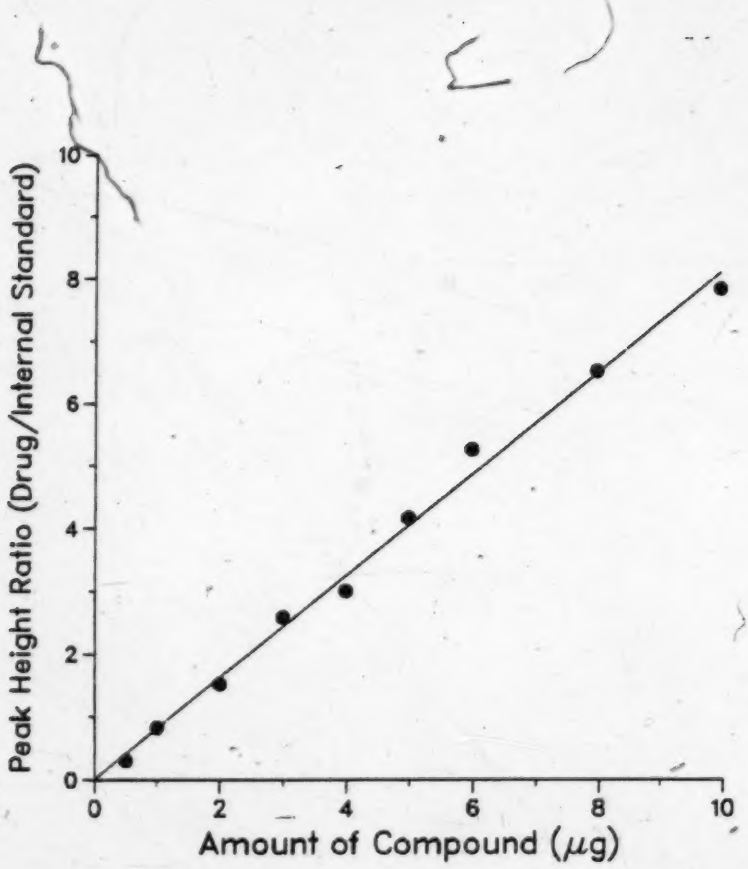

Figure III-8: Standard curve of AM in brain tissue homogenates.

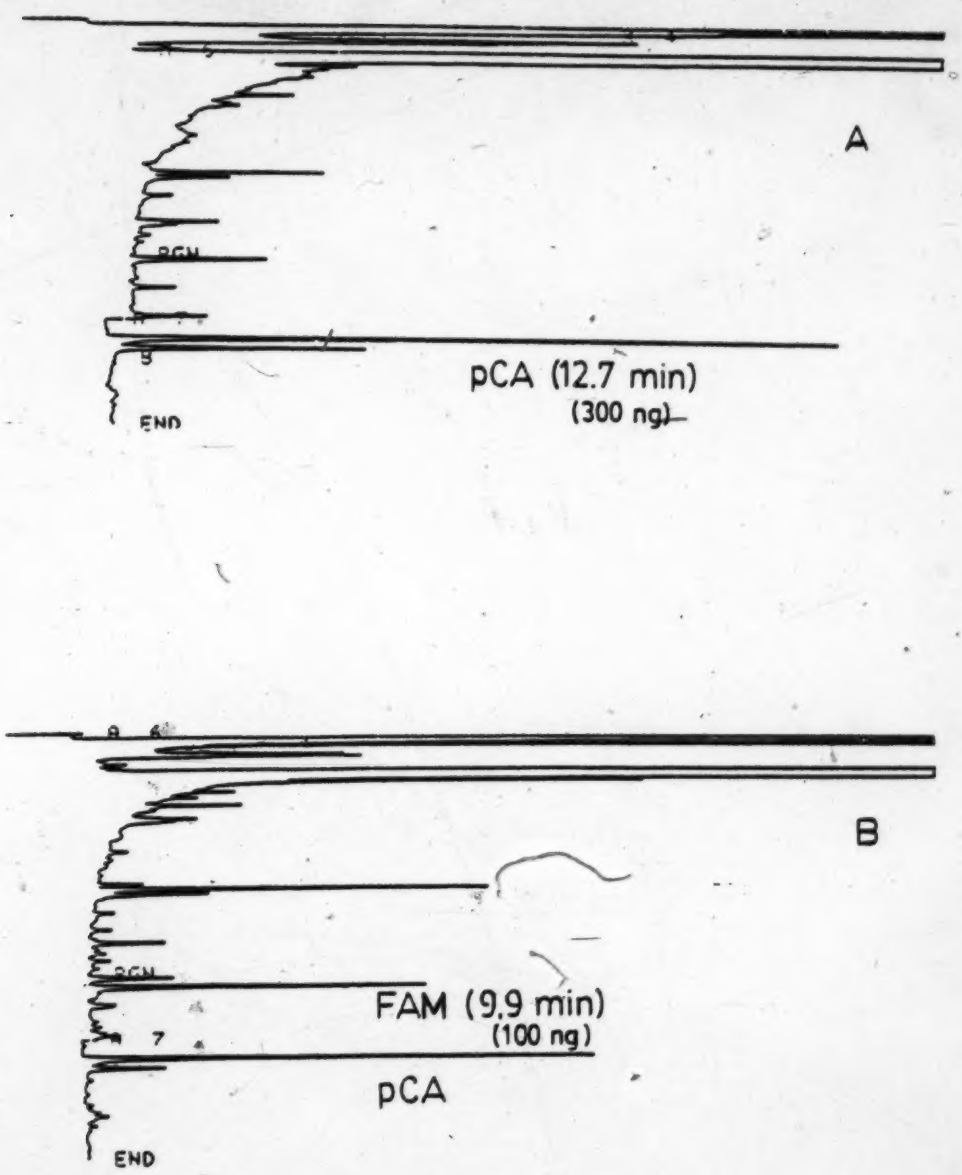

Figure III-9: Gas chromatographic traces (using 1 μm coated column) of the pentafluorobenzoyl derivatives of brain tissue homogenates of untreated (A) and FAM-treated (B) mice.

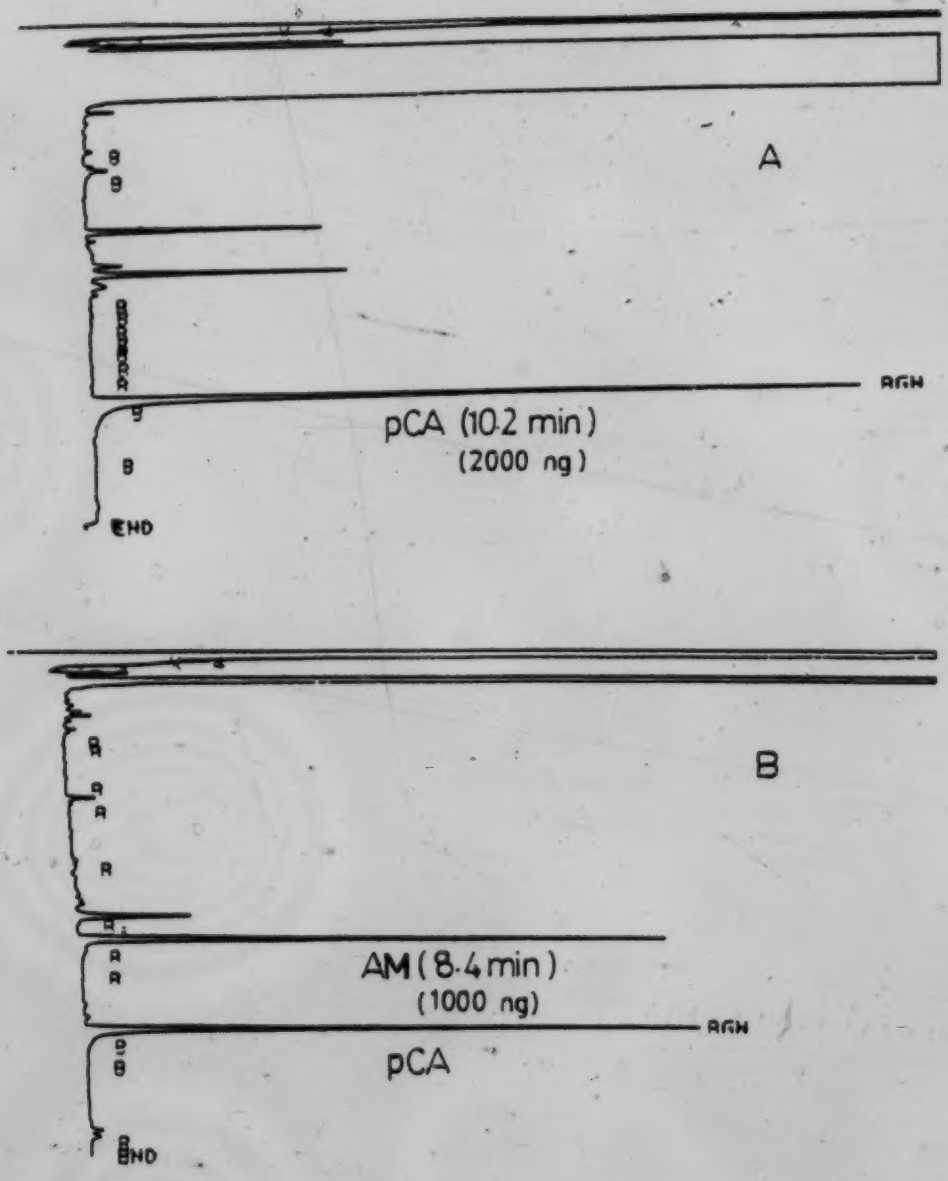

Figure III-10: Gas chromatographic traces (using 0.25 μm coated column) of the pentafluorobenzoyl derivatives of blank (A) and AM-spiked (B) murine brain tissue homogenates.

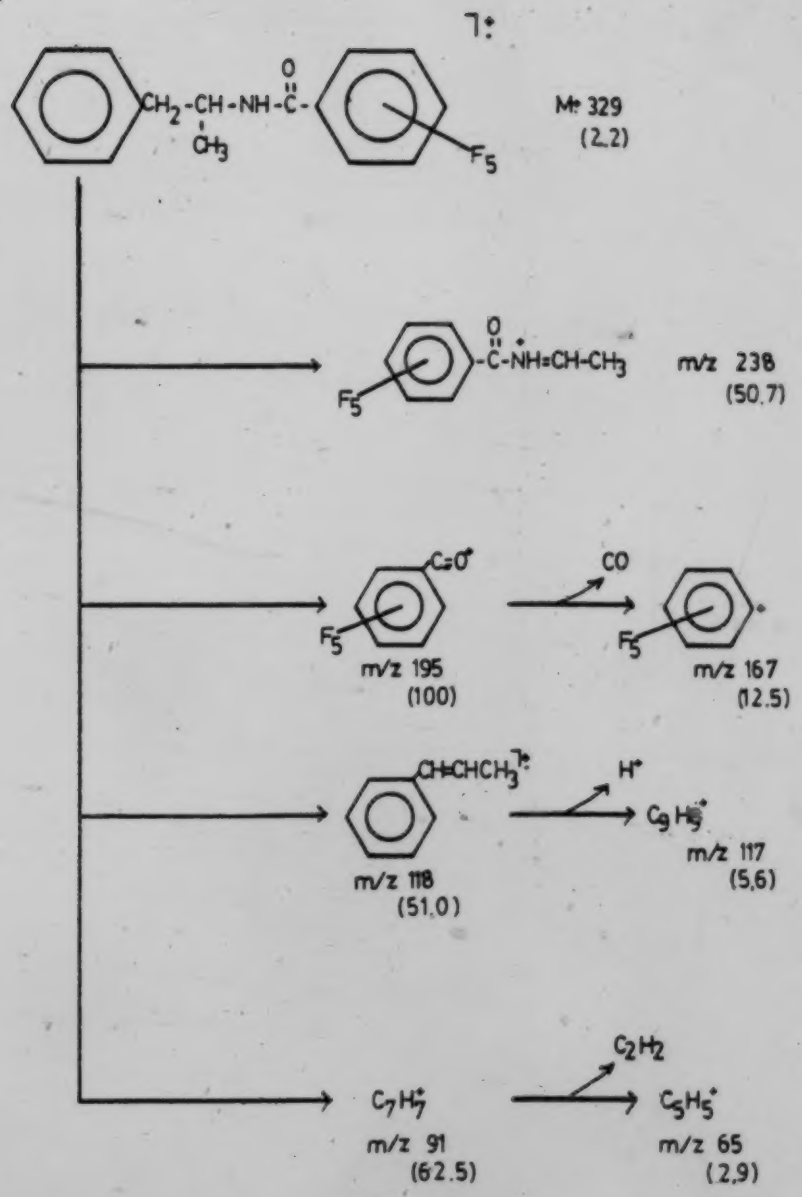

Figure III-11: Mass Spectrometric identification of the pentafluorobenzoyl derivative of AM. Numbers in parentheses represent % relative abundance.

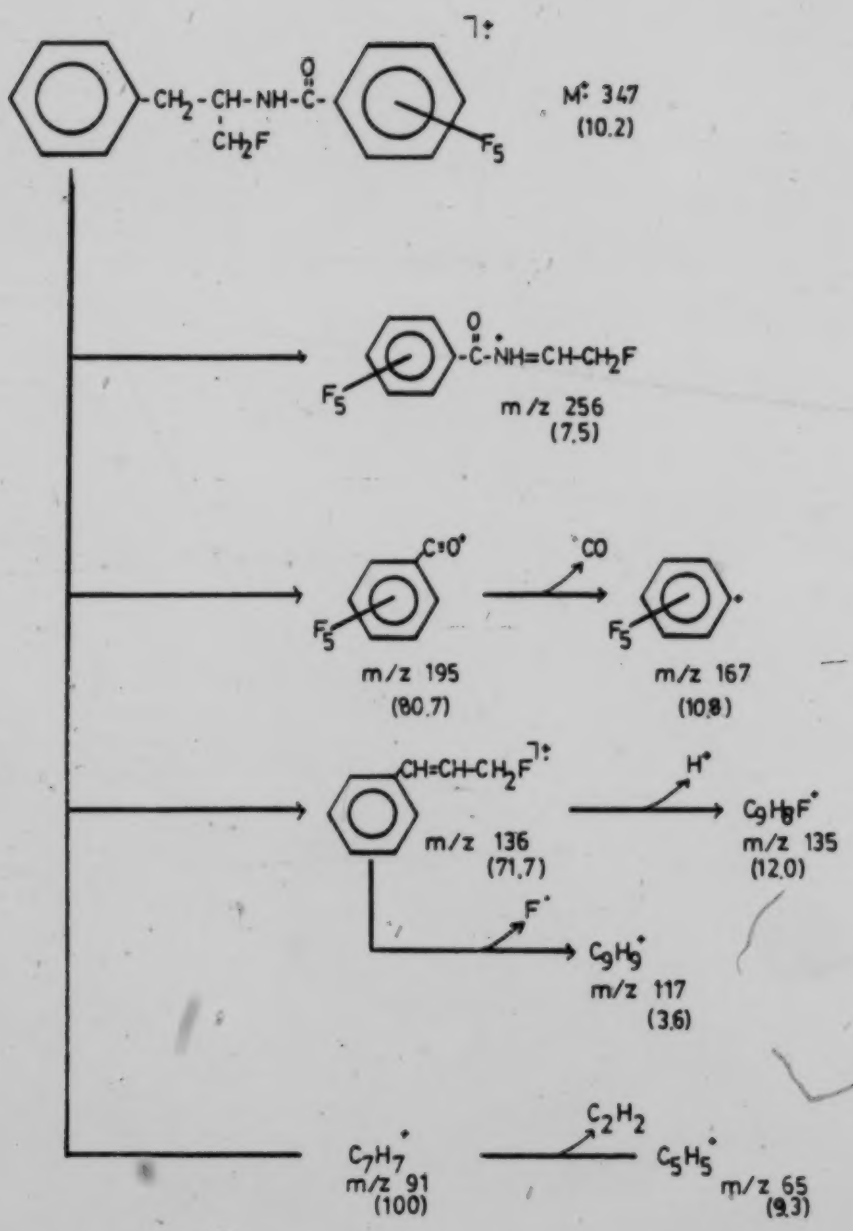

Figure III-12: Mass Spectrometric identification of the pentafluorobenzoyl derivative of FAM. Numbers in parentheses represent % relative abundance.

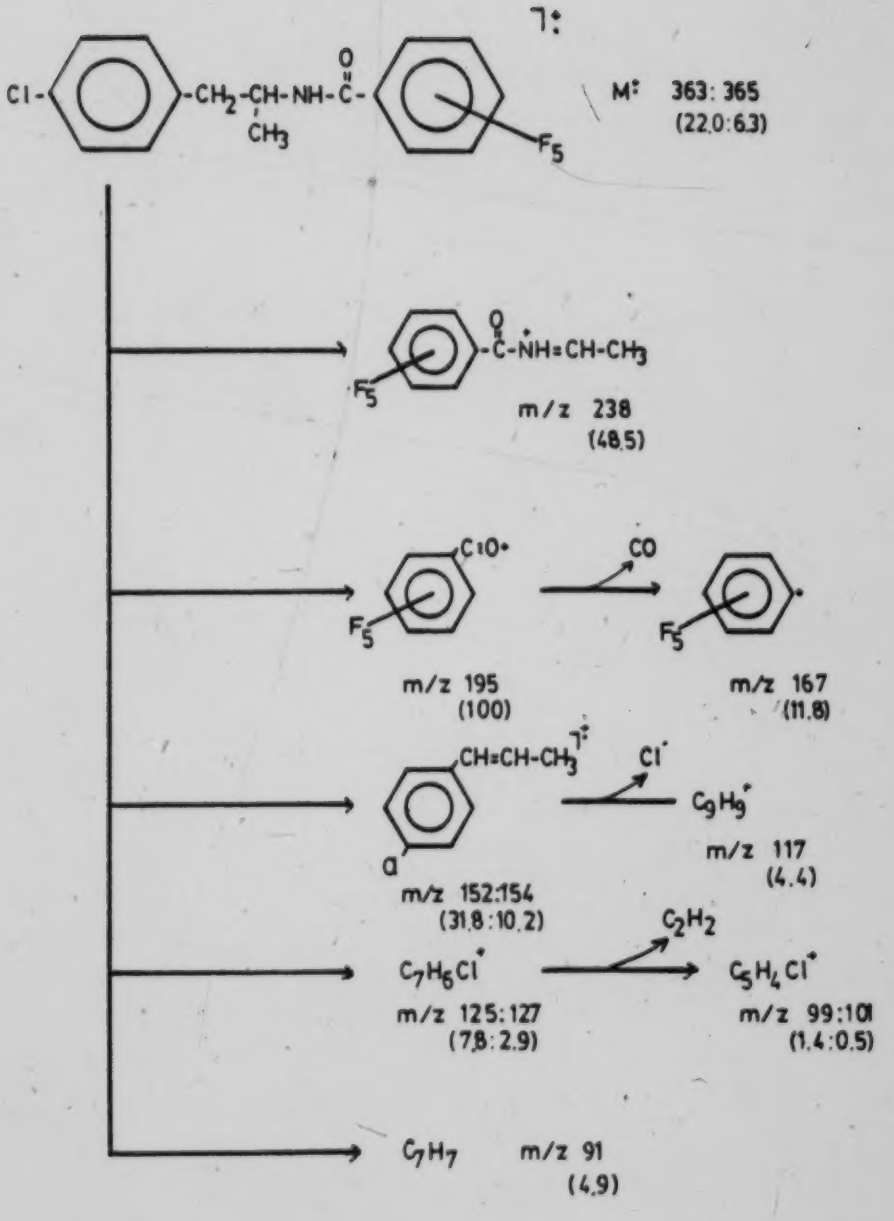

Figure III-13: Mass Spectrometric identification of the pentafluorobenzoyl derivative of pCA. Numbers in parentheses represent % relative abundance.

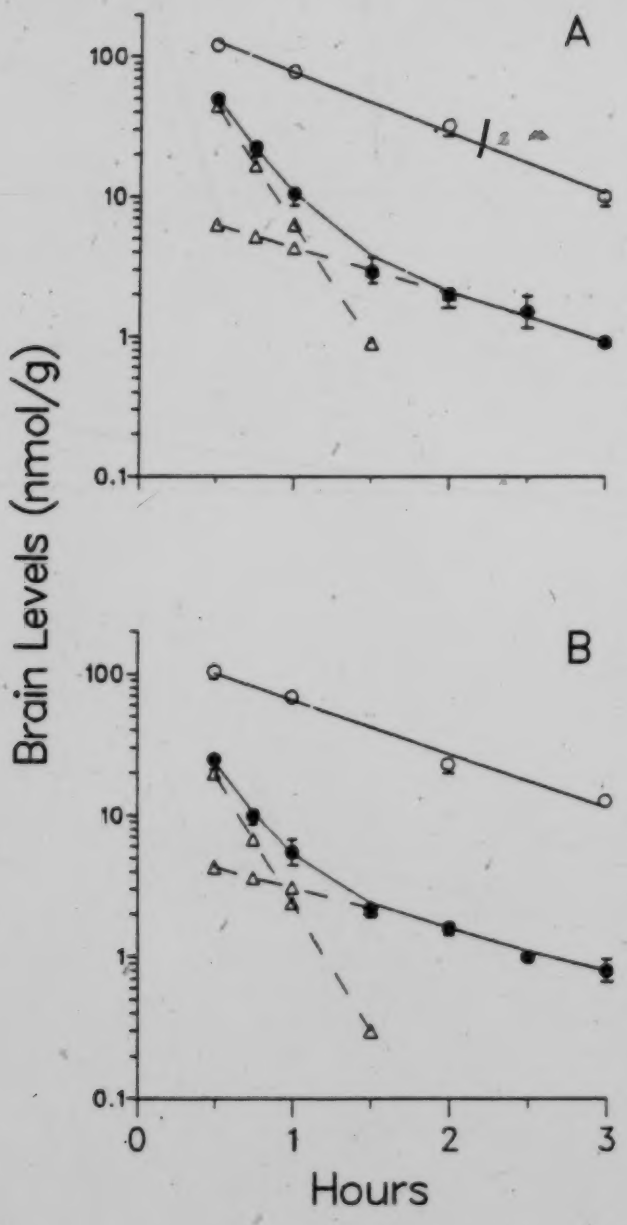

Figure III-14: Concentration of R-(+)-FAM and S-(+)-AM (A) and S-(-)-FAM and R-(-)-AM (B) in brain tissue of male Balb/cCr mice after i.p. injection of each drug (65 μmol/kg), (• FAM; o AM; --△-- residual lines; nmol/g ± S.E.M., n=4)

Table III-3: Half-lives (min) of R-(+)-FAM and S-(-)-FAM and S-(+)-AM and R-(-)-AM in brain tissue of male and female Balb/cCr mice.

	Male Mice	Female Mice
R-(+)-FAM		
1st phase	11	11
2nd phase	55	29
S-(-)-FAM		
1st phase	8	10
2nd phase	60	36
S-(+)-AM	42	—
R-(-)-AM	48	—

of each of the FAM enantiomers declined in similar biphasic manners but, at 30 and 45 min after treatment, levels of R-(+)-FAM were significantly ($p<0.05$) higher than those of S-(-)-FAM. Half-lives of FAM enantiomers, in the second elimination phase were similar to the monoexponential elimination half-lives of the AM enantiomers.

With the exception of minor differences, the accumulation and elimination of FAM enantiomers in brain tissue of female Balb/cCr mice were generally similar to those observed in males.

In female Balb/cCr mice, brain levels (Figure III-15) of the FAM enantiomers exhibited tendencies to be lower than those previously observed in male Balb/cCr mice (Figure III-14) treated with the same dose (65 μmol/kg) of FAM enantiomers. This difference was greater with S-(-)-FAM and was statistically significant ($p<0.05$) 120, 150 and 180 min after administration of S-(-)-FAM, but only achieved significance at 180 min after administration of R-(+)-FAM. Elimination of the FAM enantiomers occurred biphasically from female mouse brain and the absolute levels of R-(+)-FAM tended to be lower than those of S-(-)-FAM; however, this difference was less pronounced than previously observed in male mouse brain and reached significance ($p<0.05$) only at 45 min after treatment. Elimination half-lives for the FAM enantiomers from female mouse brain were not different from those calculated for male mice (Table III-3).

Brain levels of R-(+)-FAM and S-(+)-AM in rats declined in a log-linear manner similar to that of S-(+)-AM in mice (Fig. III-16). All slopes of the brain concentration *vs* time were similar, therefore there was no significant difference in the half-lives of S-(+)-AM and R-(+)-FAM at any of the doses used.

S-(-)-FAM Levels in Murine Brain, Fat and Plasma

Fuller[26,28,30] had found that low brain levels of his fluorinated analogues of AM were due to the preferential distribution into fat. Therefore, further experiments were conducted in two separate groups of mice in order to determine whether redistribution into fat might explain the rapid disappearance and low levels of the FAM enantiomers in mouse brain. In these

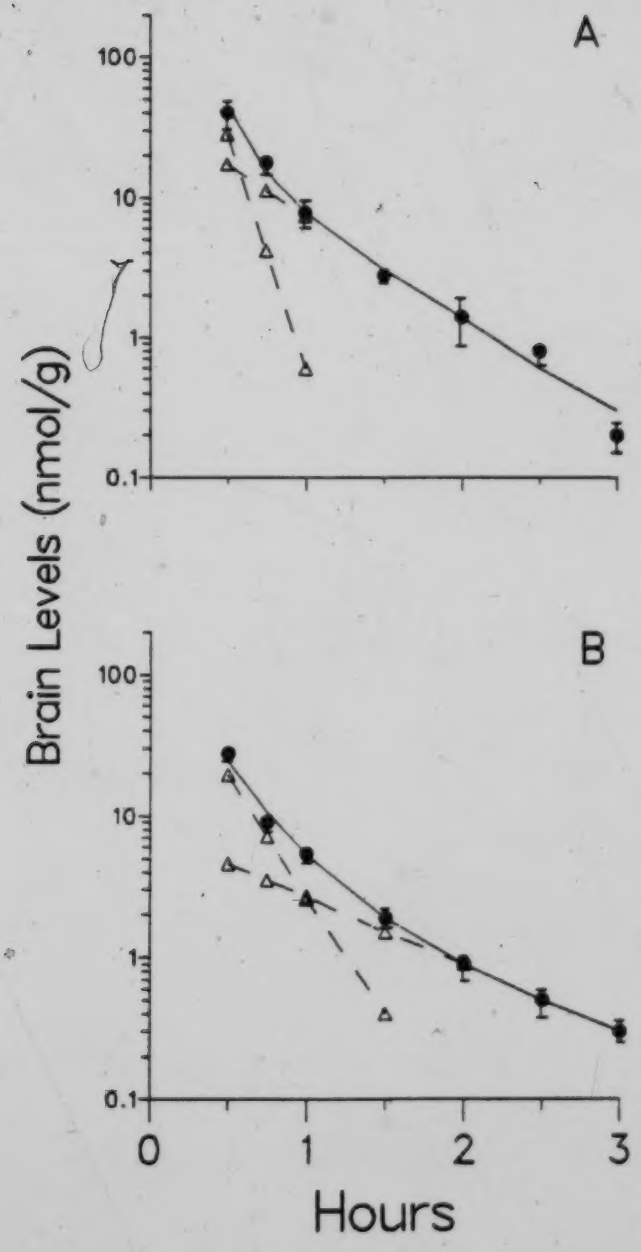

Figure III-15: Concentration of R-(+)-FAM (A) and S-(-)-FAM (B) in brain tissue of female Balb/cCr mice (n=8) after i.p. injection of each drug (65 µmol/kg). (nmol/g ± S.E.M.; --△-- residual lines)

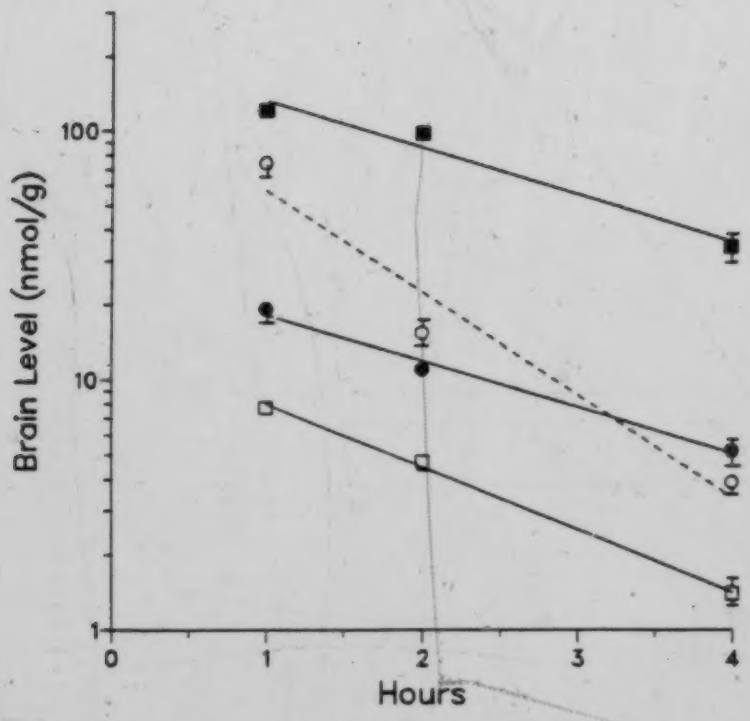

Figure III-16: Concentration of R-(+)-FAM (—) and S-(+)-AM (- - - -) (nmol/g ± S.E.M.) in brain tissue of male Sprague-Dawley rats (n=6) after i.p. injection of each drug. (FAM: □ 10 μmol/kg; • 25 μmol/kg; ■ 75 μmol/kg; AM: o 10 μmol/kg)

experiments, levels of S-(-)-FAM were measured in brain, plasma and peritoneal fat of male Balb/cCr and female ICR mice at several times after its injection (Figure III-17). although times to achieve maximum levels differed between the groups, maximum levels were attained simultaneously in each tissue and during the elimination phase maintained the rank order of brain $\geq$ fat $\geq$ plasma. Within each group, elimination from brain, fat and plasma followed parallel time courses. As previously observed in male and female Balb/cCr mice levels of S-(-)-FAM were lower in tissues obtained from female (ICR) than male (Balb/cCr) mice. Furthermore, levels of S-(-)-FAM in female ICR mouse brain were significantly ($p<0.05$) lower than those previously measured in female Balb/cCr mice (Figure III-15).

Urinary Excretion of AM and FAM by Mice and Rats

Mice treated with either enantiomer of AM excreted approximately 20% of the administered dose unchanged in a 24 h collection of urine (Table III-4). In contrast, mice treated with equimolar doses of the FAM enantiomers excreted less than 2% of the dose as unchanged drug during the same time period. No differences in the percent dose excreted were found between enantiomers of FAM, nor were there any such differences between the enantiomers of AM.

As observed with mice, rats treated with the FAM enantiomers excreted less unchanged drug in urine during a 24 h period than did rats treated with the enantiomers of AM (Table III-4). However, this difference was less pronounced than in mice and rats excreted significantly ($p<0.05$) more of a FAM dose in urine than did mice. After treatment with phenobarbital, urinary excretion of the FAM enantiomers and R-(-)-AM by rats decreased ($p<0.05$) while that of S-(+)-AM was not affected. In addition, whereas non-induced rats tended to excrete significantly ($p<0.05$) greater amounts of R-(-)-AM than of S-(+)-AM during the first 4 h after treatment, this difference was lost in phenobarbital-treated rats.

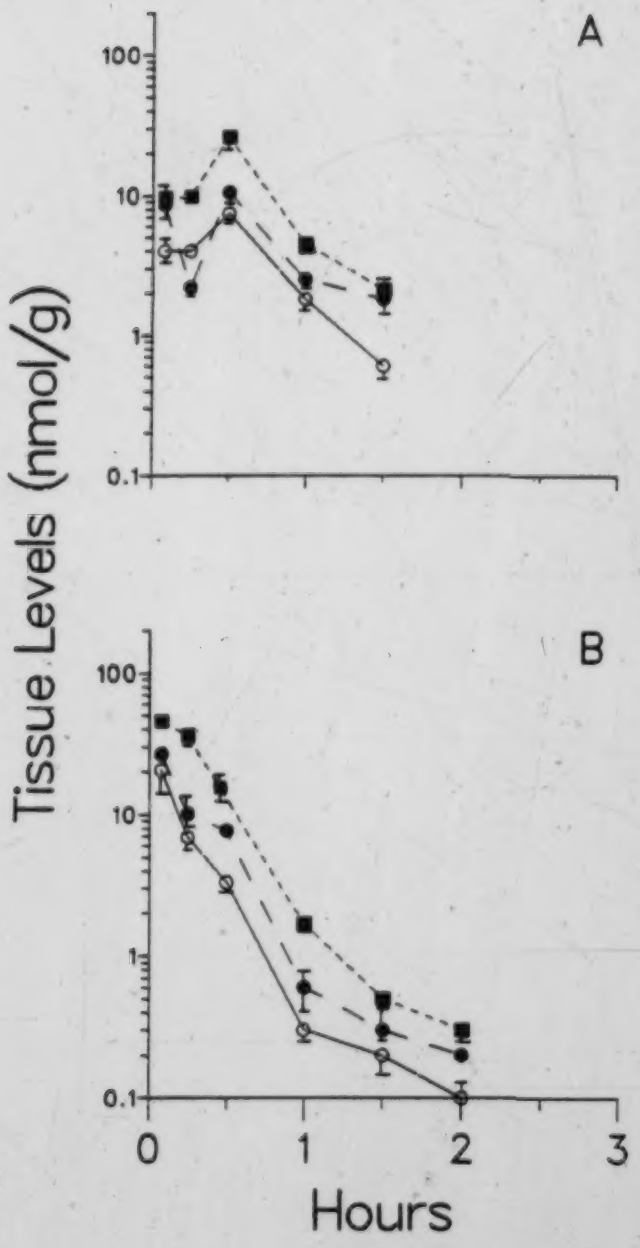

Figure III-17: Levels of S-(-)-FAM (mean ± S.E.M.) in plasma (o), fat (•) and brain tissue (■) in male Balb/cCr (A) and female ICR (B) mice (n=6) after i.p. injection (65 μmol/kg).

Table III-4: Urinary excretion of FAM and AM enantiomers by Balb/cCr Mice and Sprague-Dawley rats (n=6; mean ± S.E.M.).

	% Dose Excreted			
	0-4 h	4-8 h	8-24 h	0-24 h
Mice (dose= 65 μmol/kg)				
R-(+)-FAM	—	—	—	1.1 ± 0.3*
S-(-)-FAM	—	—	—	1.4 ± 0.2*
S-(+)-AM	—	—	—	23.3 ± 5.5
R-(-)-AM	—	—	—	22.2 ± 3.6
Naive Rats (dose= 25 μmol/kg)				
R-(+)-FAM	2.6 ± 0.5*	2.3 ± 0.6	2.1* ± 0.5	7.0 ± 1.3*†
S-(-)-FAM	3.9 ± 0.7*	1.7 ± 0.4	1.2 ± 0.1	6.8 ± 0.5*†
S-(+)-AM	6.0 ± 1.1	4.1 ± 0.8	2.4 ± 0.3	12.5 ± 1.2
R-(-)-AM	11.7 ± 1.7‡	3.3 ± 0.8	3.2 ± 1.4	18.2 ± 3.2
Phenobarbital-Pretreated Rats (dose= 25 μmol/kg)				
R-(+)-FAM	1.3 ± 0.2*	0.5 ± 0.1*	0.5 ± 0.1*	2.3 ± 0.2*†■
S-(-)-FAM	2.2 ± 0.3*	0.6 ± 0.02*	0.6 ± 0.2*	3.4 ± 0.2*†■
S-(+)-AM	7.3 ± 1.5	2.3 ± .02	3.0 ± 0.3	12.6 ± 1.4
R-(-)-AM	4.4 ± 0.8■	2.7 ± 0.4	2.4 ± 0.2	9.5 ± 1.2■

* significant difference ($p<0.05$) from AM isomer

† significant difference ($p<0.05$) from mice

‡ significant difference ($p<0.05$) from S isomer

■ significant difference ($p<0.05$) from drug naive rats

Partition Coefficients

Both the apparent and total partition coefficients were determined for R-(+)-FAM and S-(+)-AM. These enantiomers were chosen because supplies were greatest and chirality would not be expected to modify lipophilicity. The apparent partition coefficient (APC) was determined at physiological pH (pH 7.4) and not corrected for nonprotonated species concentrations. The total partition coefficient (TPC) was determined from 0.1 N NaOH. At this pH (≈ 12) both FAM and AM would exist mainly as nonprotonated species and therefore this partition coefficient would represent the actual lipophilicities of both compounds. The APC of R-(+)-FAM was significantly ($p<0.05$) higher than that of S-(+)-AM (Table III-5). However, the TPC of S-(+)-AM was significantly ($p<0.05$) higher than that of R-(+)-FAM.

C. Relation of Rectal Temperatures to Brain Levels of AM and FAM

A plot of the difference in rectal temperature (mean rectal temperature of drug-treated animals minus the mean rectal temperature of saline-treated animals) *vs* the brain levels of R-(+)- or S-(-)-FAM (Fig. III-18) in Balb/cCr mice resulted in an a loop called an hystersis. A similar plot *vs* the brain levels of S-(+)- or R-(-)-AM also resulted in an hystersis but the divergence from the hypothetical straight line was markedly less obvious than was observed with the FAM enantiomers.

Table III-5: Partition coefficients of R-(+)-FAM and S-(+)-AM (n=3; mean ± S.E.M.).

	APC[1]	TPC[2]
R-(+)-FAM	6.9 ± 0.2*	17 ± 1*
S-(+)-AM	0.16 ± 0.04	64 ± 8

* significant difference from AM

[1] Apparent Partition Coefficient

octanol: pH 7.4 buffer

$$APC = \frac{\text{amount of drug in octanol}}{\text{amount of drug in buffer}}$$

[2] Total Partition Coefficient

octanol: 0.1 N NaOH

$$TPC = \frac{\text{amount of drug in octanol}}{\text{amount of drug in 0.1 N NaOH}}$$

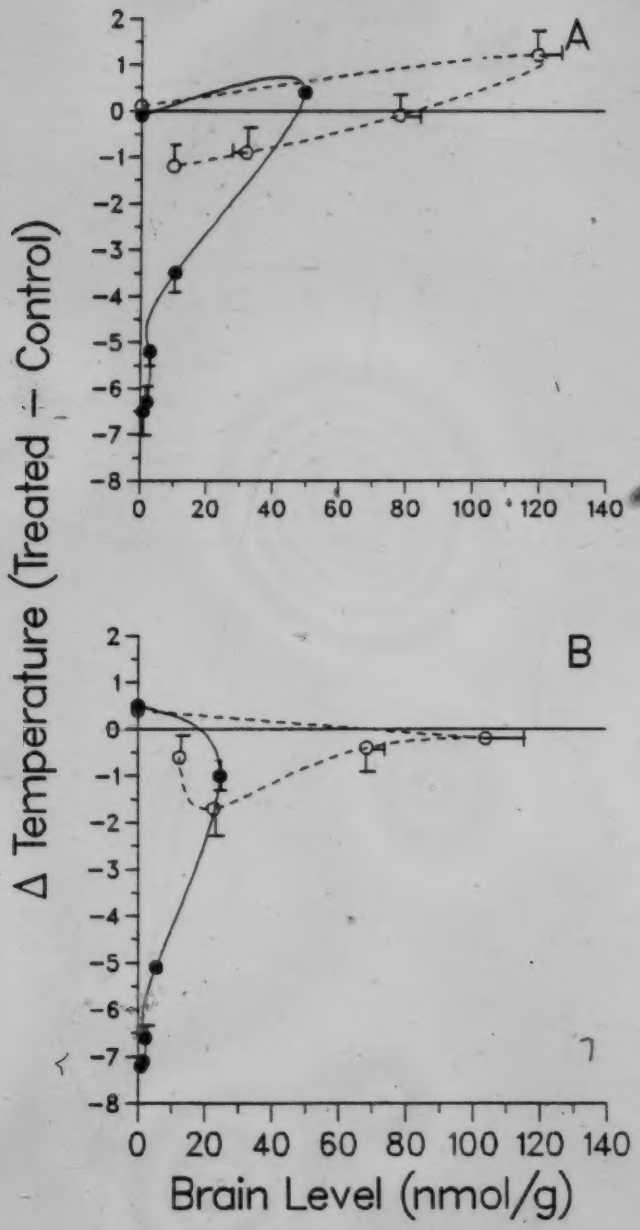

Figure III-18: Brain levels (nmol/g ± S.E.M.) of R-(+)-FAM and S-(+)-AM (A) and S-(-)-FAM and R-(-)-AM (B) *vs* the difference between the mean rectal temperatures of saline- and drug-treated Balb/cCr mice (AM, --o--; FAM, —•—).

IV. Discussion

The results support the previous work of other researchers[26,28,30,31] who found substitution of fluorine into the amphetamine side chain resulted in changes in pKa which were accompanied by modifications in the accumulation and elimination of these compounds in biological tissues. For instance, when rat brains were analysed for parent compounds one h after treatment with equimolar doses of AM, β-FAM or β,β-DFAM, the levels of AM and β-FAM were found to be similar while those of β,β-DFAM were significantly ($p<0.05$) lower.[28,29] The distribution of β-FAM was similar to that of AM (brain > fat > blood) while β,β-DFAM distributed more preferentially into fat (fat >> brain ≥ blood). The half-lives in rat brain of AM and β,β-DFAM were not different, and the β,β-DFAM was not retained longer in fat than in other tissues. In mice[30] β,β-DFAM also localized in fat more than in brain but the half-life of β,β-DFAM in murine brain was shorter than that of AM. β,β-DFAM appeared to be eliminated from brain in a log linear manner. However, brain levels were studied only at 10, 20, 30 and 40 min. It is possible that a second phase might have appeared after 40 min as happened in the case of FAM. No work was done with β-FAM in mice but it would appear that murine metabolism is more sensitive to a change in pKa than is rat metabolism. This may be because the decrease in pKa may be accompanied by a weakening of the C-N bond thus increasing the ease of deamination and thereby increasing metabolism.. However, the increase in metabolism of the β-fluoro analogue of AM compared to AM itself may be due to the higher concentration of nonprotonated species of β-FAM compared to AM at physiological pH due to the lower pKa of β-FAM. As it is the nonprotonated species which crosses the lipid-containing cell walls, it may be that more β-FAM is reaching the site of metabolism in the hepatic microsomes.

A. Locomotor Activity in Mice

Both S-(+)-AM and R-(+)-FAM produced increases in locomotor activity; however S-(+)-AM increased locomotor activity at a lower dose (33 μmol/kg) than did R-(+)-FAM (65 μmol/kg). Fuller *et al*[30] found a similar decrease in the stimulant properties of

β,β-difluoroamphetamine (β,β-DFAM). However, it was also found that equimolar doses of β,β-DFAM not only resulted in lower initial murine brain concentrations than AM, but β,β-DFAM was cleared more rapidly from the brain. As a result the dose-response curve had shifted to the right indicating that β,β-DFAM was not as potent as AM at inducing increased locomotor activity. The maximum stimulant effect was still possible but a higher dose of β,β-DFAM (160 μmol/kg) than AM (40 μmol/kg) was required to produce it. This appears to be the case for FAM as well. After equimolar doses, brain levels of both enantiomers of FAM were lower than those of AM.

Both R-(+)- and S-(-)-FAM produced decreased locomotor activity in mice. S-(+)- and R-(-)-AM also induced decreased locomotor activity in mice but, unlike the hypoactivity induced by FAM, it was of short duration and was associated with stereotypic activity. The decreased locomotor activity caused by FAM was prolonged and was associated with light sedation but no stereotypy. In other words, with FAM, the locomotor activity was replaced by sedation but with AM locomotor activity was replaced by stereotypic activity as previously discussed by Iversen and Iversen[46].

The increase in locomotor activity produced by R-(+)-FAM appears to be AM-like and may relate to the ability of R-(+)-FAM to release dopamine *in vitro*.[5,11] The extent and magnitude of the hypoactivity and hypothermia appear to be unique to the FAM enantiomers and not related to any AM-like activity.

B. Rectal Temperature in Mice

Both enantiomers of FAM produced a pronounced and prolonged hypothermia in mice. The trend toward a transient increase in rectal temperature observed in some mice immediately after treatment with R-(+)-FAM may account for the delay of onset of hypothermia observed with high doses of R-(+)-FAM. S-(+)-AM but not R-(-)-AM produced elevated rectal temperatures and increased locomotor activity in mice as has been previously reported.[58,87] S-(+)-AM at doses of 33 or 65 μmol/kg produced a significant ($p<0.05$) hyperthermic response in mice.

C. Rectal Temperatures in Rats

R-(+)-FAM at 25 μmol/kg produced elevated temperatures in the rat. Danielson *et al*[16] found that at higher doses of R-(+)-FAM (50 μmol/kg) hyperthermia was present but was followed by a decrease in rectal temperature about 3 or 4 h after treatment with R-(+)-FAM. Although the hypothermia was not as pronounced as that seen in mice, this does indicate that a biphasic temperature response can be seen in rats when the doses are high enough.

Pretreatment of rats with phenobarbital prevented hyperthermia in R-(+)-FAM-treated rats but did not eliminate the hyperthermia induced by S-(+)-AM. Although the hyperthermic effect was lost, phenobarbital pretreatment did not cause R-(+)-FAM to induce hypothermia in rats, although a lower dose induced hypothermia in mice, and a slightly higher dose (50 μg/kg) induced a delayed hypothermic response in naive rats.[16]

D. Brain Levels in Mice

In mice, the elimination of FAM from the brain is quite different from that of AM. Brain levels of the AM enantiomers are higher and more persistant than those of the FAM enantiomers. In addition, the elimination of FAM consists of two phases as opposed to AM which has one phase only. The half-lives of the second phase of FAM elimination in male mice are similar to the half-lives of AM.

The accumulation and elimination of FAM in mice appear to be isomer-, sex- and strain-dependent. Initial brain levels of R-(+)-FAM were higher than those of S-(-)-FAM in both sexes although there was no significant difference in half-lives of the FAM enantiomers between the sexes. The final brain levels of R-(+)- and S-(-)-FAM were higher in males than in females.

In addition, the brain levels of S-(-)-FAM in ICR female mice were lower at all comparable times than those of female Balb/cCr mice. This strain difference may be due to differences in absorption or metabolic rates of S-(-)-FAM. Despite these differences, all mice

showed a biphasic elimination of FAM from brain.

E. Levels of S-(-)-FAM in Brain, Fat and Plasma of Mice

The first phase of elimination of FAM from brain tissue could be due to the distribution of FAM into a distinct pharmacokinetic compartment such as fat. However, the data do not indicate that fat is a depot for FAM. Levels of S-(-)-FAM in fat were lower than levels in brain but declined in parallel to the brain and plasma levels, indicating a fast equilibration between these tissues. Therefore fat, brain and plasma appear to belong to a single pharmacokinetic compartment.

F. Urinary Excretion of AM and FAM by Mice

The rapid elimination of FAM from mouse brain does not appear to be due to preferential distribution into fat. However, the rapid elimination of FAM from the blood could simply reflect a rapid excretion from the body. Amphetamines are generally excreted through the kidneys,[78] therefore if rapid excretion is the cause of the rapid disappearance of FAM, one would expect a large percentage of the administered dose to be found unchanged in the urine. The results do not support this possibility as less than 2% of an administered dose was found in a 24 h collection of urine from mice treated with S-(-)- or R-(+)-FAM. This is approximately one-tenth of the amount of R-(-)- or S-(+)-AM excreted unchanged in a 24 h collection of mouse urine after an equimolar dose.

The remaining possible explanation for the rapid elimination of FAM from mouse tissue is that FAM is subject to rapid metabolism. Results are not available on the identification and concentration of FAM metabolites. However, Fuller[30] found β,β-difluorination of AM resulted in a compound (β,β-DFAM) which was more rapidly metabolized by mice than AM itself.

G. Brain Levels of AM and FAM in Rats

In rats, after treatment with equimolar doses, the brain levels of R-(+)-FAM were significantly ($p<0.05$) lower than those of S-(+)-AM at 1 h but not at 2 or 4 h after treatment. The semi-log slopes of the brain level *vs* time curves were not significantly different between S-(+)-AM and R-(+)-FAM, therefore the half-lives were similar. As well, the half-life of R-(+)-FAM in rat brain was similar at all doses although there appears to be a trend to a shorter half-life at the lowest dose (10 μmol/kg). Fuller *et al*[30] have shown in rats that β,β-DFAM is metabolised by deamination while AM is metabolised mainly by *para*-hydroxylation. It may be R-(+)-FAM is also metabolised by deamination.

In comparison with mice, rats treated with R-(+)-FAM had higher brain levels at comparable times and the elimination of FAM from rat brain was in a log-linear manner with a half-life longer than that observed in mice.

H. Urinary Excretion of AM and FAM by Rats

Rats excreted significantly ($p<0.05$) more FAM intact in urine than did mice, but the amount excreted was still less than 10% of the administered dose. As observed with mice, rats also excreted significantly ($p<0.05$) less FAM than AM in urine. This may be due to a higher tubular reabsorption of FAM. Although the total amount of FAM excreted in 24 h was significantly ($p<0.05$) lower than the amount of AM excreted, the only collection in which FAM was significantly ($p<0.05$) less than AM was the first 4 h collection. In comparison, it was found that the brain levels of AM and FAM, after equimolar doses, were significantly ($p<0.05$) different only at 1 h and not at 2 and 4 h. These differences may reflect a more rapid metabolism of FAM compared to AM, however, this is not supported by the data as the half-lives of elimination of AM and FAM from rat brain tissue were similar. The observed dissimilarity between FAM and AM may be due to differences in the absorption or distribution of the two compounds.

The excretion of both FAM enantiomers was significantly ($p<0.05$) reduced at all collection times after phenobarital pretreatment which may indicate deamination is one of the

metabolic pathways of FAM. Fuller *et al.*[31] reported that pretreating rats with phenobarbital reduced the half-life of β,β-DFAM in tissues but did not effect the half-life of AM. The conclusion was β,β-DFAM followed a different metabolic pathway (deamination) than AM (*para*-hydroxylation). The deamination pathway was inducible by phenobarbital as supported by an increase in α-carbon oxidation and N-oxidation products *in vitro* after treatment with phenobarbital. The excretion of S-(+)-AM was not affected by pretreatment with phenobarbital. In the first 4 h of urine collection, naive rats excreted significantly ($p<0.05$) more R-(-)-AM than S-(+)-AM; this isomer difference has been previously noted.[18] In enzyme-induced rats this difference was lost; the excretion of R-(-)-AM decreased significantly ($p<0.05$) in the first 4 h so there was no longer a significant difference in the amounts of AM enantiomers excreted. This may relate to previous findings that R-(-)-AM is a better substrate for deaminating enzymes than S-(+)-AM.[14,31] It is possible that the phenobarbital-induced pathway is also available to R-(-)-AM.

I. Partition Coefficients

The low lipophilicity (as demonstrated by the low TPC) of FAM may account for the fact that fat does not act as a preferential site of uptake for FAM. At physiological pH FAM would exist as both the protonated (76%) and nonprotonated (24%) forms compared to AM which would exist mainly in the protonated form (99%). It is the neutral form which would be absorbed by fat, however, the neutral FAM molecule is less lipophilic (ie. less fat-soluble) than the neutral AM molecule. It appears that the larger number of neutral FAM molecules is not enough to overcome the decreased lipid solubility, thus FAM distribution in mouse tissue is similar to the AM distribution observed by Fuller.[30]

J. Relation Between Brain Levels of AM and FAM and Rectal Temperature in Mice

Drug-induced physiological changes can be either directly or indirectly related to drug concentration. In a direct relationship, a particular concentration of drug will always produce the same intensity of effect regardless of whether that concentration is attained during the

absorption or elimination phase. In comparison, agents which exhibit indirect relationships between effect and concentration will not produce equally intense physiological effects at equivalent concentrations achieved during the absorption and elimination phases.

These possibilities can be graphically represented as in Figure IV-1,A-E. Figure IV-1,A represents overlapping plots of drug concentration for two hypothetical agents which exhibit identical time courses. In this plot, drug concentrations attained at time 1, during the absorption phase, are equal to the concentrations attained at time 3, during the elimination phase. Although the tissue levels of these two agents may exhibit the same time course, the time courses of the physiological effects may not be indentical. Figures IV-1,B and D represent two possible time courses for the physiological effects produced by these agents. In a direct relationship, the time course of physiological response parallels the drug concentration time course. Thus, at times 1 and 3, when tissue levels of drug are the same (Figure IV-1,A), the intensities of the physiological response are also the same (Figure IV-1,B). If effect is plotted against concentration, points 1 and 3 converge (Figure IV-1,C) and all points lie on a straight line passing through zero. If an indirect relationship exists between drug concentration and physiological effect, the time course of the physiological effect will not parallel that of the drug concentration, as indicated by comparison of Figures IV-1,A and IV-1,D. Under these circumstances, although the drug concentrations are identical at times 1 and 3 (Figure IV-1,A), they do not produce equivalent physiological responses (Figure IV-1,D). As a consequence, when effect is plotted against concentration, points 1 and 3 diverge and a loop (hystersis) rather than a straight line results (Figure IV-1,E).

Comparison of Figures III-4 and III-14, suggest that the FAM-induced temperature changes do not parallel brain levels of FAM. Indeed, when temperature changes were plotted against brain concentrations of FAM, an hystersis resulted (Figure III-18), further supporting that an indirect relationship exists between these parameters.

Such hysterses (Figure IV-1) can result when:

1. the parent compound is responsible for the observed effect but there is a delay in reaching the active site.[43,44] For example, it may be that the hypothermic effect is related to the

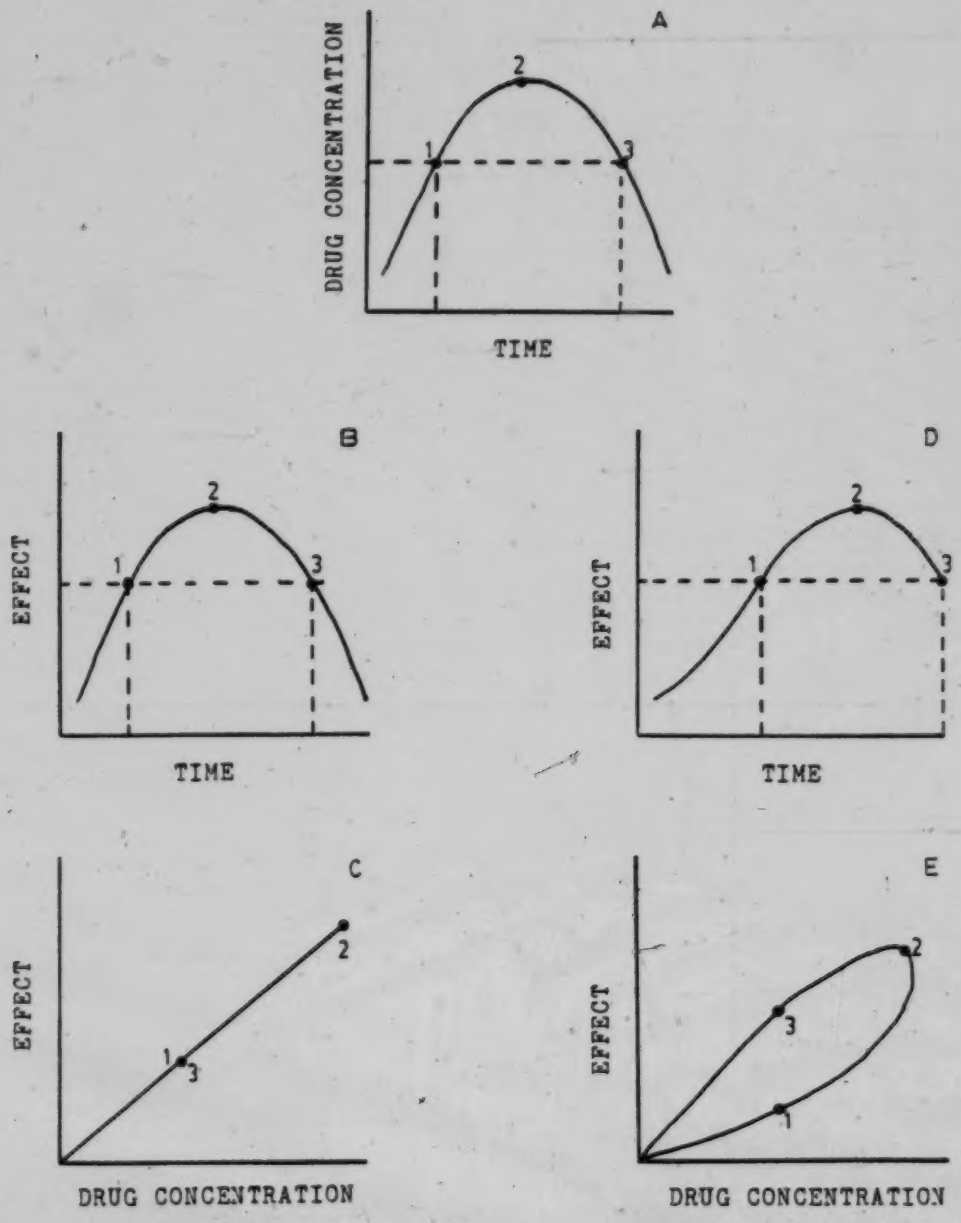

Figure IV-1: Theoretical effect vs drug concentration relations.

concentration of FAM in a particular area of the brain (eg. the hypothalmus) rather than to whole brain levels. If so, there could be a delay in attaining effective concentrations in this area which could be masked by the whole brain levels. To determine whether this is the case it would be necessary to determine FAM levels in brain areas.

2. the parent drug is responsible for the observed effect but the observed effect is due to an indirect mechanism of action.[44] For example, plasma warfarin levels do not directly correlate with prothrombin time. However, warfarin changes the time to clotting through an indirect method, that is it limits the availability of Vitamin K, thereby limiting formation of the prothrombin complex.[44] If plasma warfarin levels are compared to the decrease in the activity of the Vitamin K-dependent reaction there is a direct correlation. In the case of an indirect action, the observed changes may not be the best measure of effect.
3. the parent drug is not responsible for the observed effect which is due to an active metabolite.[43] If this is the case, then plotting observed effect *vs* the concentration of the metabolite would result in a linear relation. To determine whether this situation applies to FAM, it would be necessary to first identify the possible metabolites and then attempt to relate their concentrations to the observed changes. As the decrease in locomotor activity and rectal temperature is maximum at low brain levels, the theory of an active metabolite is appealing. As this decrease is not isomer-specific, it may be the active metabolite, if it exists, has lost the chiral centre and therefore both enantiomers of FAM could produce the same active metabolite.

There is another possible explanation why the maximum temperature decrease occurs when brain levels are low. Perhaps FAM is stored in neuronal vesicles (as has been found with *p*-hydroxyamphetamine[47,48]) or bound to a membrane and released slowly over an extended period of time. The first phase of FAM elimination could be the rapid metabolism of excess compound while the second phase reflects the slow release. The rapid onset of hypothermia (at 65 μmol/kg S-(-)-FAM, rectal temperature decreases 7.5° in 1.5 h) could be due to the initial high concentrations of FAM while the comparatively slow recovery of normal rectal

temperature (increase of 1.5° in 4 h) could reflect the slow release of FAM either from storage vesicles or receptor sites.

K. Comparison of FAM Effects in Mice and Rats

When the temperature responses of mice and rats to R-(+)-FAM are compared, there appears to be a species difference in the effect of R-(+)-FAM on rectal temperature. Mice treated with R-(+)-FAM up to 65 μmol/kg show only decreased rectal temperatures while rats treated with 25 μmol/kg exhibit hyperthermia. This may be due to the difference in metabolism of the two species. As has been previously mentioned, rats metabolize AM predominately by *para*-hydroxylation while mice metabolize AM by deamination as well as *para*-hydroxylation.[7,8,76] Thus, hypothermia may be due to a metabolite produced by the mouse but not produced by the rat except at higher doses.

This could reflect possible differences in the deamination mechanism of these two species. There appear to be two possible routes to deamination of AM: α-carbon oxidation (α-C oxidation) or N-oxidation.[6,7] α-C Oxidation involves the hydroxylation of the carbon α to the amino group with a subsequent loss of the amino group to produce a ketone or alcohol. N-Oxidation appears to involve the direct hydroxylation of the nitrogen of the amino group, producing oximes, and may lead to the loss of the amino group. Support for N-oxidation as a route to deamination is based on the correlation between a species ability to N-oxidize chlorphentermine (an AM analogue with a *p*-chloro substituent to prevent *para*-hydroxylation and an added α-methyl group which prevents deamination).[6,7] Interestingly, neither mice nor rats produce N-oxidation products from chlorphentermine. In rats this is understandable as this species metabolizes AM mainly by *para*-hydroxylation. However, mice are capable of deaminating AM. Therefore the studies with chlorphentermine indicate that mice must deaminate *via* α-C oxidation rather than through N-oxidation.

In *in vitro* studies with rat hepatic microsomes, Fuller[31] found that β,β-DFAM was metabolized at a greater rate than AM. In addition, the major metabolite (~80%) produced from β,β-DFAM was the oxime (an N-oxidation product) compared to AM which produced

alcohol and ketone (α-C oxidation products) in approximately equal amounts. When hepatic microsomes from phenobarbital-treated rats were used, both AM and β,β-DFAM showed increased production of metabolites although the proportions of α-C and N-oxidation products remained similar to those found with naive rat liver microsomes. It should be noted that, in both studies, the total amount of metabolites resulting from β,β-DFAM was higher than that from AM.

If a metabolite is responsible for the production of hypothermia in mice and rats (at high doses), it may be that more of the metabolite is produced by mice. This would explain why mice exhibit FAM-induced hypothermia to a greater extent than rats. If the hypothermia is due to an α-C oxidation metabolite (which would be achiral), mice would be expected to produce more of this metabolite than rats as mice appear to favour α-C oxidation while rats favour N-oxidation. Mice also metabolize FAM more rapidly than rats and could produce more achiral metabolite and therefore exhibit hypothermia at lower doses than do rats.

L. Summary

The present data indicate that substitution of a fluorine atom into the α-methyl group of AM results in a compound with striking pharmacologic, behavioral and pharmacokinetic differences from AM. However, these data do not distinguish whether the unique pharmacologic and behavioral properties of FAM are due to the parent compound or to a metabolically-derived agent. Further studies of the metabolism of FAM thus seem to be warranted.

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